ADVANCES IN THE CONTROL OF ZOONOSES

BOVINE TUBERCULOSIS
BRUCELLOSIS — LEPTOSPIROSIS
Q FEVER — RABIES

WHO/FAO Seminar on Zoonoses

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INTRODUCTION

This monograph contains the principal papers which were presented, and summaries of the discussions which took place, at the WHO/FAO Seminar on Zoonoses held in Vienna from 24 to 29 November 1952 This meeting was sponsored jointly by the Regional Office for Europe of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) of the United Nations Its purpose was to bring together medical and veterinary officials of European countries to discuss certain diseases of public health and economic importance in this region

The term ' zoonoses", although fairly new in the vocabulary of public-health and veterinary medicine, is a useful one to denote diseases of animals transmissible to man A report of a Joint WHO/FAO Expert Group on Zoonoses * deals with the subject in general and devotes special attention to five of these diseases Annex 1 of this report lists more than 80 zoonoses, but as regards European countries, perhaps 10 or 15 can be said to occupy a position of importance The five diseases considered at the Seminar were selected for their broad interest to the countries of Europe

The papers were prepared with the knowledge that both administrative officers and laboratory workers would attend the meeting It would obviously have been impossible, within the time limits imposed, to prepare comprehensive reviews on each of the topics, and emphasis was therefore placed rather on the highlights and recent advances with respect to each disease The discussions which followed each section are here presented in very summary form no more than the salient points raised at the meeting are indicated FAO and WHO will be glad to supply further technical information upon request

There can be little doubt as to the usefulness of bringing together medical and veterinary workers—whether their work lies in administration, in the laboratory or in the field—to discuss topics of common interest Particularly does this apply to the zoonoses, where a combined medical and veterinary approach, utilizing the full resources of public health and agricultural departments, constitutes the most effective means of combating these diseases

* *World Hlth Org techn Rep Ser* 1951 40

The prevention and eradication of zoonoses in human beings can be accomplished in large part by control of these diseases in animals, so that it is natural for public health officials to give every assistance—moral, financial, scientific, and educational—to agricultural authorities in carrying out animal disease control programmes. Practically speaking, this can take the form of financial subsidies in, say, bovine tuberculosis and brucellosis control programmes; the fostering of research and epidemiological surveys in newly emerging problems such as are presented by Q fever and leptospirosis; popular education in the control of rabies and hydatidosis; and many other projects, indicated in the papers which follow. The contribution which can be made by agricultural and veterinary authorities in tracking down the source of infection (the animal), applying measures for control and eradication, co-operating in surveys, and educating the farmers, is obvious.

To integrate the efforts of public health and agricultural authorities, a development in recent years has been the establishing of veterinary public health units in many countries, usually within the organizational framework of municipal, district or federal departments of health. These units act as the bridge connecting public health and agricultural resources and interests, and serve to synthesize the attack on the various problems of concern to each group. Their size and their degree of active operation in the field vary in different countries according to local conditions. The scope of veterinary public health is broader than that of zoonosis control—comprising in addition, for example, food hygiene. More information on this subject will be found in the report of the Joint WHO/FAO Expert Group on Zoonoses referred to previously.

It is hoped that this seminar has encouraged closer co-operation between public health and agricultural authorities, so that accelerated strides can be made in lessening the public-health and economic toll exacted by the zoonoses.

Part I

BOVINE TUBERCULOSIS

BOVINE TUBERCULOSIS IN MAN AND CATTLE

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Tuberculosis in man is produced by two types of the tubercle bacillus, the human and the bovine—a fact which may well have stamped the epidemiology of this disease to a high degree [13]

The problems associated with the typing of the human and bovine tubercle bacilli are far-reaching, but here space permits of only a brief survey of the matter

Type Determination

Two methods of typing are available in one the morphology of the colonies serves as the basis for classification, the other method is based on the virulence of the given strains to the rabbit and the guinea-pig By using a suitable culture medium [3] it is possible with great certainty to differentiate the dysgonic bovine type from the human type Löwenstein's [illegible] of the colonies of the two types on this medium is very pronounced

Fig 1 and 2 show the characteristic morphology of colonies of the eugonic human type and the dysgonic bovine type The colonies are of the same age

In table I an outline is given of the properties of the two types

By combining these two methods we have found [11] that a great majority of the strains of tubercle bacilli isolated from man can be classified with a high degree of certainty as either typical human or typical bovine strains

In the course of our typing of tubercle bacilli isolated from about 5,500 patients, however, we have found several strains which deviate from the two well defined types [5-10, 12] These atypical strains may be divided into subgroups

primary culture will often show a few eugonic colonies, and, if the culture is under observation sufficiently long, it will be noticed that numerous buds arise from the dysgonic colonies and that subsequently these buds may completely overgrow the mother colonies Subcultures from these buds show a typical eugonic growth On determination of the virulence of these dysgonic human strains we find the same virulence as for typical human strains

Attenuated bovine and human strains[7]

These strains are found almost exclusively in cases of lupus On this account, primary cultures should always be made in this disease, if guinea-pig inoculation alone is employed, many of the attenuated strains will be lost

Here we meet with the problem of how we are to classify the attenuated strains of tubercle bacilli Firstly, they may be divided into two groups The first group grows as typical bovine colonies Only a few of these are of standard virulence for the rabbit and guinea-pig, most of them being attenuated, on account of their virulence for these animals, however, the strains may be referred to the bovine type, although a few of them are so attenuated for both species that they may be typed only by estimation of the morphology of their colonies That these attenuated dysgonic lupus strains are bovine has been shown by Stanley Griffith who, by rabbit passage, was able to increase their virulence so that their typing became practicable This has been our experience, also If such strains are typed merely after the outcome of their inoculation into rabbits, they will be recorded erroneously as belonging to the human type Eugonic lupus strains may be either virulent for the guinea-pig and avirulent for the rabbit—that is, typically human—or more or less attenuated for the guinea pig The strongly attenuated eugonic lupus strains must be designated "typeless", since they may equally well be attenuated human strains or attenuated eugonic bovine strains

When typing a large amount of material we not infrequently meet with cultures made up of a mixture of bovine and human tubercle bacilli,[9, 10] this "mixed infection" is found most often in patients with pulmonary tuberculosis Its frequency is more strikingly illustrated in patients suffering from pulmonary tuberculosis produced by the bovine type, when type determination allows us to ascertain how many cases become mixed infections In this way we have demonstrated that of 17 cases of bovine pulmonary tuberculosis examined over more than one year, 9 developed into mixed infections—a change which has rarely occurred within the first year of examination In a few cases we observed that the bovine type ultimately disappeared and was replaced by the human type Table II shows one of the most thoroughly examined cases in this series

TABLE II DEMONSTRATION OF MIXED INFECTION IN ONE PATIENT BY VARIOUS METHODS*

Date of sputum examination	Number of colonies	Percentage of eugonic colonies	Date of sputum examination	Number of colonies	Percentage of eugonic colonies	Percentage of eugonic colonies in Besredka medium	Demonstration of bovine type by intravenous injection of 5 mg Besredka culture into rabbit
30 7 36	∞	10	19 1 38	403	94	56	
29 9 37	431	9.5	26 1 38	492	98	—	
4 10 37	338	55	2 2 38	432	99.5	47	
12 10 37	553	7.8	9 2 38	308	100	84	
20 10 37	650	0	16 2 38	382	98.4	92	
27 10 37	273	50	23 2 38	646	99.5	—	
3 11 37	403	16	2 3 38	335	100	—	
11 11 37	461	98	9 3 38	352	100	—	
19 11 37	356	77	9 4 38	391	100	—	
24 11 37	410	85	10 5 38	350	100	100	+
2 12 37	595	91	10 6 38	489	100	—	
8 12 37	110	97	9 7 38	660	100	—	
15 12 37	569	71	14 9 38	331	100	100	+
22 12 37	144	81	12 11 38	510	100	—	
5 1 38	374	84	13 12 38	762	100	98.5	+
12 1 38	386	92	23 3 39	520	100	89.3	+

* Inoculation on Löwenstein's medium with 0.75% glycerol

From this table it is evident

(*a*) that the number of bovine tubercle bacilli decreases to such an extent that cultures from sputum on Löwenstein's medium showed the human type in pure culture at the last 10 examinations,

(*b*) that *cultures from* sputum in Besredka's medium subcultured on Löwenstein's medium may be able to demonstrate the bovine type when the primary Löwenstein culture fails to do so,

(*c*) that by inoculation of rabbits with 5 mg of primary Besredka culture it was still possible to demonstrate the presence of the bovine type in the two cases in which this type could not be demonstrated by methods (*a*) and (*b*)

These mixed infections are mentioned because, *in the hands of the* less experienced examiner, they may cause some disagreement between the mo [illegible] Apart from th [illegible] beings [illegible] not be taken into consideration

Epidemiological Relationships

Tuberculous infection may occur in two forms (1) alimentary infection, which is caused almost exclusively by the bovine type, and (2) inhalation infection, which, while it is caused chiefly by the human type, may be produced *also by the bovine type, especially among the rural population* in districts where tuberculosis is prevalent in cattle

Alimentary infection

The alimentary infection arises from consumption of milk that contains bovine tubercle bacilli It is generally assumed that the infection which occurs through the intestinal canal is considerably more benign than the inhalation infection [16] This does not, however, apply to infants Indeed, we know that in districts where tuberculosis is frequent among cattle, tuberculous meningitis and cervical adenitis caused by the bovine type are not rare among *infants (see tables III, IV, and VII)*

Most often the alimentary infection does not result in progressive tuberculosis, nor does it produce any symptoms, manifesting itself only in the positive tuberculin reaction Indeed, in some districts in Denmark where tuberculosis among cattle was formerly very common, we have found *the percentage of children with a positive tuberculin reaction to* be considerably higher than in other districts where the cattle were free

TABLE III OCCURRENCE OF TUBERCULOUS MENINGITIS AND MILIARY TUBERCULOSIS IN FOUR COUNTIES 1925-32*

County	Age-group						Total	Case-rate per year per 10 000 inhabitants	Case-rate for udder tuberculosis per year per 10 000 cows
	0-5	5-10	10-15	15-25	25-50	over 50			
Bornholm	4	4	2	6	2	1	19	0.5	0.8
Lolland-Falster	36	18	11	15	17	1	98	0.9	5.3
Tønder	22	5	4	3	3	2	39	1.3	30.0
Ringkøbing	67	32	16	32	20	6	173	1.4	23.0

* After Bindslev

TABLE IV OCCURRENCE OF CERVICAL GLAND TUBERCULOSIS IN FOUR COUNTIES 1925-32*

County	Age-group							Total	Case-rate per year per 10 000 inhabitants	Case-rate for udder tuberculosis per year per 10 000 cows
	0-5	5-10	10-15	15-25	25-50	over 50	unknown			
Bornholm	1	0	1	2	13	1		18	3.9	0.8
Lolland-Falster	11	11	8	31	24	10	1	96	7.2	5.3
Tønder	2	3	9	17	4	7	1	43	11.3	30.0
Ringkøbing	22	27	44	103	68	22	15	301	19.2	23.0

* After Bindslev

TABLE V RESULTS OF TUBERCULIN TESTING IN PERSONS IN CONTACT WITH CATTLE IN THE SAME RURAL DISTRICT*

Age (years)	Results for persons in contact with			
	tuberculous herds		tuberculin-negative herds	
	number of persons tested	percentage showing positive tuberculin reaction	number of persons tested	percentage showing positive tuberculin reaction
0-5	61	18.0	121	1.6
6-10	128	48.1	90	3.3
11-15	125	64.8	61	9.8
16-20	53	71.8	—	—
21-35	8	98.3	—	—
Over 35	212	99.5	—	—

* After Table 69 in Sigurdsson[14]

from tuberculosis Similarly, persons in contact with tuberculous herds were found to be more often infected than persons in contact with tuberculin-negative herds (see table V)

In one county with a high incidence of cattle tuberculosis (Haderslev), among children in the age group 7-8, 47% in the towns and 23 3% in the rural districts showed a positive tuberculin reaction, while at 14-15 years the figures were 74 8% and 60 9% respectively In another county (Bornholm), where no tuberculosis among cattle had been noted for a long time, the corresponding figures for positive tuberculin reactions were 6 8% in the first age-group for both urban and rural districts, and 15 5% and 12 8% in the second (see table VI)

TABLE VI RELATION BETWEEN INCIDENCE OF CATTLE TUBERCULOSIS AND RESULTS OF TUBERCULIN TESTS FOR URBAN AND RURAL POPULATIONS *

Age (years)	Tuberculin test Bornholm county (no cattle tuberculosis)				Tuberculin test Haderslev county (much cattle tuberculosis)			
	towns		rural district		towns		rural district	
	number of persons tested	positive tuberculin reactions (%)	number of persons tested	positive tuberculin reactions (%)	number of persons tested	positive tuberculin reactions (%)	number of persons tested	positive tuberculin reactions (%)
7 8	222	6 8	296	6 8	768	47	343	23 3
8-9	320	10 0	520	6 7	918	51.3	388	31 6
9 10	336	8 6	474	6 1	984	59 9	410	33 9
10-11	279	13 3	479	9 8	1,022	62 3	410	37.3
11 12	327	16 8	529	10 0	1 068	63 3	402	45 9
12 13	372	17 7	498	10 4	1 080	68.5	397	51 8
13 14	385	12 5	479	12.3	1 116	70 4	403	57 1
14 15	323	15 5	407	12 8	842	74 8	166	60 9
15 16	294	24 1	387	13 4	598	74 1	—	—
16 17	295	18 3	422	18 0	611	77 0	—	—
17 18	315	20 6	422	16 8	484	74 6	—	—
18-19	280	26 1	383	18 0	298	73 0	—	—
19 20	237	27 8	340	20 3	108	72 5	—	—
20-21	230	37 4	327	26 9	—	—	—	—

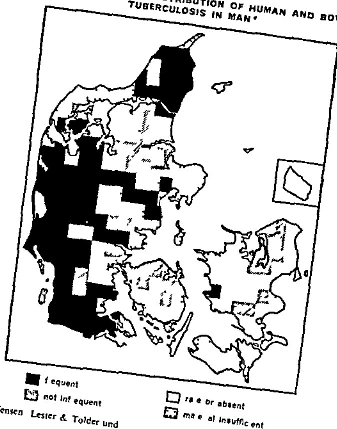
* After Madsen Holm & Jensen [31]

Inhalation infection

In the inhalation infection the human type tubercle bacillus plays a predominant role Recent investigations have shown, however, that in districts where tuberculosis is prevalent among cattle, the bovine type has a not altogether insignificant part, since about 50% of the cases of pulmonary tuberculosis arising among inhabitants on farms with tuberculous cattle were found to be caused by the bovine type [11 15]

The inhalation infection almost invariably arises in stables—and I should like to emphasize that working in highly infected cow stables has to be looked upon as being far more dangerous than working in a tuberculosis hospital We have examined the water used for washing the windows and walls of such stables and have often found bovine tubercle bacilli in no small quantity

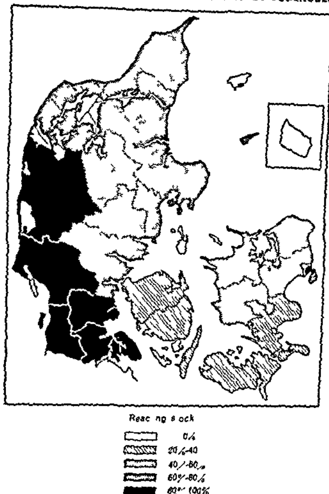
FIG 4 GEOGRAPHICAL DISTRIBUTION OF HUMAN AND BOVINE TUBERCULOSIS IN MAN*



* From Jensen Lester & Tolder und

Fig 4 and 5 show the distribution in Denmark of the human and bovine types of tuberculosis in man in 1930 6 and the geographical distribution of cattle tuberculosis in 1935 Comparison of the two maps reveals a fairly close concordance Unfortunately our material does not allow of a more detailed presentation as the number of type-determined cases is too

FIG. 5. GEOGRAPHICAL DISTRIBUTION OF CATTLE TUBERCULOSIS*



* From Jensen, Lesse & Tolderlund.

small to justify conclusions concerning individual localities (shown as squares in fig. 4) nor does our knowledge of the distribution of tuberculosis among cattle by any means permit of a more detailed differentiation. However, in order to investigate the difference in frequency of the bovine infection between the rural and the urban populations, our material was divided into the following groups: (1) towns in Jutland, (2) rural districts in Jutland, (3) towns on the islands, (4) rural districts on the islands, (5) Bornholm, (6) Copenhagen (see tables VII and VIII). Division into these large groups naturally reduces the inaccuracy implied in the geographical localization

TABLE VII. FREQUENCY OF BOVINE INFECTION AMONG URBAN AND RURAL POPULATIONS*

Strain isolated from	Age-group											
	0-5 years			5-15 years			15-30 years			over 30 years		
	human type	bovine type	per-centage bovine type	human type	bovine type	per centage bovine type	human type	bovine type	per-centage bovine type	human type	bovine type	per-centage bovine type
	towns in Jutland											
Sputum	10	1	9.1	27	2	6.9	240	11	4.4	58	2	2.9
Cervical glands	1	5	83.3	3	10	76.9	8	10	55.6	22	5	18.5
Spinal fluid	14	5	26.3	6	3	33.3	15	1	6.3	2	0	0.0
	rural districts in Jutland											
Sputum	15	4	21.1	45	13	22.4	363	75	17.1	186	16	7.9
Cervical glands	0	6	100.0	4	24	85.7	23	32	58.2	28	8	22.2
Spinal fluid	6	23	79.3	26	16	38.1	45	14	23.7	14	1	6.7
	towns on the islands excepting Bornholm											
Sputum	15	0	0.0	17	0	0.0	98	0	0.0	33	0	0.0
Cervical glands	0	0	—	0	0	—	4	1	—	0	0	—
Spinal fluid	5	3	37.5	5	2	28.6	9	0	0.0	4	0	—
	rural districts on the islands, excepting Bornholm											
Sputum	15	1	6.3	38	2	5.0	275	13	4.5	144	1	0.7
Cervical glands	1	1	—	1	13	92.9	7	4	36.4	16	1	5.9
Spinal fluid	5	5	50.0	5	2	28.6	10	3	23.1	14	0	0.0
	Bornholm											
Sputum	2	0	—	18	0	0.0	52	1	1.9	27	0	0.0
Cervical glands	2	0	—	1	0	—	1	0	—	4	0	—
Spinal fluid	0	0	—	0	0	—	0	0	—	0	0	—
	Copenhagen											
Sputum	351	28	6.9	225	12	5.1	888	16	1.8	348	2	0.6
Cervical glands	4	10	71.4	3	19	86.4	29	15	34.1	30	2	6.3
Spinal fluid	68	12	15.0	28	6	17.6	29	1	3.3	15	2	11.8

* After table 5 in Jensen Lester & Tolderlund [1]

of individual cases Bornholm is considered separately because tuberculosis in cattle on this island has been completely eradicated Copenhagen is also recorded separately because many of its inhabitants—especially the children—in their vacation visit other parts of the country, and thus may acquire the bovine infection anywhere outside Copenhagen

Relation between alimentary and inhalation infections

As mentioned before, the relatively mild alimentary milk infection gives rise to a great many cases of latent symptom free infection, which merely brings about a positive tuberculin reaction Since th... may be interpreted as a sign of interest... and the organism ... against subseq...

TABLE VIII FREQUENCY OF BOVINE INFECTION IN CASES OF SURGICAL TUBERCULOSIS AMONG URBAN AND RURAL POPULATIONS*

District	Age-group											
	0-5 years			5-15 years			15-30 years			over 30 years		
	human type	bovine type	% bovine type	human type	bovine type	% bovine type	human type	bovine type	% bovine type	human type	bovine type	% bovine type
Towns in Jutland	7	5	41.7	6	5	45.5	59	10	14.5	51	8	13.6
Rural districts in Jutland	9	7	43.8	28	24	46.2	132	67	32.7	128	24	15.8
Towns on the islands excepting Bornholm	2	0	—	4	1	—	26	3	10.3	30	3	9.1
Rural districts on the islands excepting Bornholm	4	2	33.3	13	3	18.8	52	7	11.8	52	3	5.5
Bornholm	2	0	—	3	0	—	10	0	0	4	0	—
Copenhagen	13	3	18.8	15	3	16.7	102	8	7.3	62	5	7.5

* After table 5 in Jensen, Lester & Tolderlund

We noted that in some districts the eradication of tuberculosis among cattle was soon followed by a considerable fall in the percentage of positive tuberculin reactions among children. The question therefore naturally arose whether it might not be expedient to refrain from eradication of tuberculosis among cattle, thus retaining the more benign alimentary infection and inducing a mass immunization which later might protect the population against the far more serious inhalation infection.

The energetic combating of man to man transmission has resulted in a reduction of the tuberculosis mortality in Denmark between 1880 and 1940 to about one seventh its former magnitude. We were thus placed in a difficult situation. The bovine inhalation infection was rather widespread among the rural population, and if we failed to combat tuberculosis among cattle we should run the risk of having the human infection replaced by the bovine. It would, in fact, be the rural population who would have to pay the greater part of the price for a possible induction of mass immunity in the entire population. It is obvious, indeed, that in a modern society such a costly method of immunization must be said to be antiquated.

A vigorous campaign has therefore been conducted against cattle tuberculosis, which is now completely eradicated in Denmark,[1, 4] and since

the percentage of positive tuberculin reactions was found to be steadily decreasing, particularly among children, a comprehensive Calmette vaccination of the population has been under way since 1938 [5, 13]

Human-type infection in cattle

The problem raised by transmission of infection from man to cattle is of considerable significance in the eradication of bovine infection in both man and cattle Investigations made by veterinarians [11] in Finland and Denmark have shown that a human infection in cattle may give rise to a temporary positive tuberculin reaction although, except in very rare cases, no tuberculous processes are demonstrable in the animals If the source of the infection is removed, the reactivity usually disappears within about half a year Cows infected with the human type of bacillus do not transmit the disease to man whereas the control of bovine tuberculosis is greatly complicated by the presence of the human infection in cattle

Bovine type infection of cattle by man

After it had been demonstrated that pulmonary tuberculosis in farmers and farmhands was frequently caused by the bovine tubercle bacillus, the question arose whether such patients could transmit the infection to the cattle In 1940 Nielsen & Plum [14] reported no less than 17 herds infected by the transmission of the bovine tubercle bacillus from man In all these cases the infection was transmitted to herds which previously were tuberculin negative and direct or indirect infection from animals could practically be excluded With all 17 herds it was found that the person who had infected them was suffering from pulmonary tuberculosis caused by the bovine type

Thus, there can be no doubt that intensive co-operation between veterinarians and physicians is of the utmost importance, and in this connexion I should like to make the following remarks

(1) Physicians should report to the Veterinary Directorate every case of pulmonary tuberculosis caused by the bovine bacillus In Denmark this is quite an easy matter, as tubercle bacilli from all tuberculous patients are cultured at the Statens Seruminstitut, Copenhagen

(2) Tuberculosis dispensaries should assist veterinarians in examining everyone working on a farm where reactors suddenly appear and where it is not possible to demonstrate any other source of infection

(3) Frequent occurrence in any district of tuberculosis of the cervical lymph nodes and tuberculous meningitis in children is a sign of widespread cattle tuberculosis The attention of veterinary surgeons should be called to this point

(4) In Sweden milk epidemics caused by tubercle bacilli of the bovine type have been reported In the search for the cause of such epidemics, as close a co operation between the veterinary authorities and physicians should be established as is advocated when a streptococcal milk epidemic is being investigated

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* Consultation of the bibliographies of some of these references will provide a more comprehensive guide to the literature particularly to the non Danish work

CONTROL OF BOVINE TUBERCULOSIS IN CATTLE

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Tuberculosis among cattle has presented a problem to veterinarians in every country in the world, not only because of the losses which are caused by the disease but because the infection is capable of setting up active disease in man, frequently involving glands, bones, and joints, and not uncommonly occurring in the lungs

It is very difficult to assess the losses which arise among cattle and other livestock The general finding is that when pigs, horses, or even sheep are infected, they have acquired disease from contact with cattle, only occasionally does infection in other species create a hazard to bovines In herds of cattle, the disease takes a heavy toll The animal may in fact succumb after a protracted period during which it fails to thrive, or the disease may progress rapidly, as when haematogenous spread occurs and acute miliary tuberculosis develops There are, therefore, obvious direct losses from the disease However, there is a heavy depreciation in animals in which the disease assumes a more chronic form because they become less efficient in consequence as milkers, breeders, or feeders Cows in which the uterus becomes involved in the disease process will produce tuberculous calves, and in many cases tuberculosis is a contributing factor in infertility in a herd

The incidence varies considerably, and some countries have already eradicated the disease In Great Britain it was estimated in 1931 that about 40% of cows were infected In 1945 it was calculated that some 17% to 18% of cattle would probably be "reactors", this would represent some 30% to 35% of cows At this time the incidence was about 20% in England, 14% in Scotland, and 7 5% in Wales, so that it is clear that infection is not evenly distributed throughout the country (The figures have been markedly reduced since then, as progress has been made in establishing tubercle free herds, and the incidence in the country as a whole is probably [1952] between 10% and 12%) The weight of infection varies within wide limits both in herds and localities, depending on the type of herd, the system of herd management, and the traditional cattle trade in the district The most heavily infected areas are usually near industrial districts where

the cattle population is dense and intensive milk production is practised, with close housing of cattle, often in "flying" herds[1] which rear few or no young stock Where the cattle population is sparse, notably in hill country, the incidence may be very low There is no doubt that the practice of housing cattle in cowsheds which are not well lit or well-ventilated favours the spread of infection, and that rearing cattle under range conditions contributes very largely towards control of the disease

The detection of tuberculous cattle is the most important question to be considered in formulating any method of control or eradication of the infection Clinical examination of the animals is of some value, and it is possible to identify many affected animals in this way An animal will require careful examination if there is a history of rapid decline in condition following calving, such indefinite symptoms as general unthriftiness, listlessness, some loss of milk, or a degree of infertility, or if it shows more obvious signs such as accelerated respiration, frequent cough, or induration with or without enlargement of a quarter of the udder Close examination should always include auscultation of the lungs, which will very often provide the necessary evidence It is sometimes an advantage to drive an animal out of the cowshed and force it to exercise before the examination is made As additional and most important aids to diagnosis, samples of milk, sputum, vaginal or uterine discharge, and urine may be examined microscopically, and, particularly in the case of milk, may, if negative, be submitted to biological test It is frequently possible, therefore, to diagnose the disease when there is (*a*) considerable lung involvement, (*b*) infection of the udder, (*c*) infection of the uterus and occasionally the kidneys, (*d*) disease in lymph-glands which are palpable, notably the retropharyngeal glands

The identification of animals affected in this way has considerable advantage in that it may be possible to remove them from the herd (in Great Britain they are slaughtered compulsorily with payment of compensation) and thus reduce the weight of infection in milk, with obvious benefit from the public health point of view Removal of these "open" cases is of great importance if the farmer is proposing to eliminate disease from his herd, unless other active steps are taken, however, it will not make any significant contribution to the reduction of infection in the herd, since the majority of diseased animals are infective long before they can be recognized as "open" cases Many infected animals, even with extensive disease, may show no recognizable symptoms, and, even after careful examination, infection cannot be diagnosed by the methods available to the veterinary surgeon

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[1] Flying herds are those in which new animals are constantly being introduced and sold and there is a continual change of population within the herd

The only method of detecting tuberculous cattle for the control or eradication of the disease is the tuberculin test, while other methods have no place in modern schemes of eradication, it is still necessary in any system of eradication depending on the tuberculin test to identify the dangerously infective animal so that it may be removed at once from contact with other cattle

The Tuberculin Test

In the first place it is essential to see that the tuberculin used is of sufficiently high potency to ensure that it will elicit a response in a very high proportion of tuberculous animals otherwise, any scheme of eradication is doomed to failure The potency of the tuberculin depends on its content of tubercle-protein and it is now possible by using purified protein derivative tuberculin not only to ensure that potency is adequate but to maintain a constant potency

Tuberculin may be used as a diagnostic agent, but the test in cattle must be used with discrimination, and properly interpreted Many different tests have been employed, but at the present time the intradermal test in one form or another is usually applied This test depends on the fact that a tuberculous animal is sensitized to tuberculin, and that a local reaction develops at the site of inoculation This allergic response is not specific because sensitivity may be produced by a variety of organisms The bovine type of the organism is the one which produces the serious disease process in cattle The human and the avian types may occasionally establish lesions in cattle and will certainly produce sensitization to tuberculin, the avian type more frequently sets up lesions *Mycobacterium johnei*, which causes Johne's disease, the organisms associated with so-called skin tuberculosis, the vole bacillus and BCG, likewise sensitize to tuberculin, and it may be that other related organisms are also capable of producing this sensitivity

Thus even with a sufficiently potent tuberculin it is not an easy matter to decide on the significance of a positive tuberculin test response While it is generally true that the homologous tuberculin may be expected to give the best response—for example, a tuberculin produced from the bovine type of organism will give the best response in an animal infected with the bovine type—there is a wide variation in the size and quality of specific reactions So much so, that animals sensitized by organisms other than the bovine type of *Myc tuberculosis* may give positive results which cannot be differentiated from many of those found in infected animals

As a result, a positive reaction in an individual animal of unknown history is of comparatively little significance Further tests may have to be made to establish a diagnosis, but the test must not be repeated too soon or too often, otherwise the animal may fail to give any appreciable response, since there is evidence that even in infected animals there is frequently a reduction in sensitivity, and therefore in the response to tuberculin The test is, however, of much greater value when an entire herd of known history is tested, and its history taken into account The presence of cases of clinical tuberculosis, lesions of skin tuberculosis, or Johne's disease, should all be taken into account It is usually advisable at the first test of a herd to diagnose bovine tuberculosis in all those animals which give a response to mammalian tuberculin, if there are a number of frankly positive reactors in the herd, as in practice it is found that it is much more profitable to ensure removal of all infected animals at the earliest possible stage

In order to distinguish between animals infected with bovine type tuberculosis and those sensitized by other organisms, the comparative test has been developed This involves the use of two tuberculins, mammalian and avian, which are injected in separate sites , the results of each reaction are noted The test is applied on a herd basis, and the interpretation of the result in individual animals depends on the general picture in the herd as a whole The presence of bovine infection may be established by clinical examination of animals, and this will mean that the test results are interpreted strictly, and animals showing marked responses, even if the avian response is very similar, will be rejected as reactors Enquiry must be made about the possibility of the presence of Johne's disease, and lesions of skin tuberculosis must be sought The fact that some animals respond only to avian tuberculin may be accepted as evidence of the presence of a non specific infection This will mean that the test result is interpreted more liberally and that only those animals in which the mammalian response is considerably in excess of the avian will be classed as reactors Other animals with a less marked excess mammalian reaction will be classed for further test, as will those which give a minor response to mammalian tuberculin and a negative reaction to avian In the test in such a herd, animals with a small mammalian excess reaction, and all those with an avian excess reaction, even if there is a positive response to mammalian tuberculin, will be accepted as being sensitized by an organism other than the bovine type

When animals are retested by the comparative test it is the usual practice to carry out the retest rather more than a month after the first Generally, one may expect the homologous tuberculin to give a better response at retest than the heterologous, for the reaction to the heterologous tuberculin will have decreased to a greater extent

Eradication from a Herd

It has already been said that the incidence of disease in a herd depends to a very great extent on the method of husbandry. If a herd is self-contained, that is to say, if the adult replacement-cattle are bred within the herd, the incidence of infection may not be high unless the conditions of housing and management are particularly unsatisfactory, allowing infection to spread rapidly. If the young stock are in close contact with the adult stock, infection may be more or less evenly distributed throughout the herd. If the young stock (calving heifers, heifers, and calves) are maintained apart from the cows, the former are often comparatively free of infection; a large number of reactors found among the young stock in these circumstances may result from infection from the milk of a cow with tuberculosis of the udder, and one or two reactors among the young stock may have been born from cows with tuberculosis in the uterus. The heifers usually become infected when they join the adult herd. The incidence of infection in the herd is likely to bear some relation to the number of replacement cattle which have to be purchased. The "flying" herd, for which replacements are constantly purchased in the open market, is usually heavily infected.

The procedure which must be followed in eradicating the disease from a herd is decided after the result of the first tuberculin test is known. It may vary with the incidence of infection among the various groups of stock already mentioned.

If there are few reactors, they should be removed and short interval tests made until infection is eliminated.

If reactors are too numerous to allow the farmer to remove them at once, he may eliminate the disease more gradually. In this case the reactors and non reactors are segregated, after removal of any animals showing clinical evidence of disease. Calves born from the infected portion of the herd are immediately placed with the non reacting portion. By keeping the two lots of animals separate it is possible gradually to build up the tubercle free section, while reactors are disposed of as opportunity offers, and replacements for reacting cows become available from among the non-reacting heifers. This system is, of course, most satisfactory if separate farms are available, and is less successful when the farm buildings and grazings are not suitable for maintaining two separate lots of cattle in virtual isolation.

When a group of animals, usually the cows, shows a large proportion of reactors (particularly if an "open" case of tuberculosis has been found among them), it is often good practice to remove the whole of the heavily

infected group and to replace it by purchased tubercle free stock The less heavily infected groups can be retained after reactors have been eliminated from them This method is practicable only if there are numbers of tubercle free replacements available, but it does provide a useful outlet for healthy cattle surplus to breeders' requirements which might otherwise be sold into herds in which no progress is being made It eliminates the disappointments which arise at successive tests when the farmer has decided to retain non-reactors from a heavily infected group, because many of them will react at subsequent tests, having probably picked up infection about the time of the first test, or having been in a non-allergic state, though infected, at that time It allows the owner of the herd to obtain the benefits of tubercle-freedom at an early stage Many farmers, particularly in Scotland, have followed this system, and the herds have subsequently shown a highly satisfactory history

Much of what has been proposed depends on the facilities which exist for the disposal of reactors from herds In some countries it has been possible for the animals to be slaughtered with compensation to the owner under an official scheme In others the incentive to proceed to eliminate disease has been indirect, by the offer of bonus payments which accrue only after infection has been eliminated or by some form of financial aid provided through farmers' co operative societies In any event, it seems that in spite of the benefits which arise from the improvement in health and productivity in herds free of disease (and often from the better prices obtainable for tubercle-free stock), it is usually necessary to encourage eradication by the provision of some kind of financial inducement to farmers to cover the immediate cost of tuberculin testing and elimination of reactors Perhaps the best method of doing this is under an official scheme, when the whole process of eradication can be controlled and conducted in an orderly fashion, using the best methods available

It is most important, when eradication of the disease is proceeding in a herd by segregation and removal of reactors, to ensure that the buildings occupied by the reactors are properly cleaned and disinfected Cleaning of walls, floors, and fittings with a hot 4% solution of sodium carbonate before disinfection is recommended, indeed, thorough scraping and cleaning with this solution is a much greater precaution than actual disinfection Manure made by the reacting stock is either removed direct to arable land or stacked away from contact with cattle Fields on which reactors have grazed are left vacant for at least a month Adequate cleaning and disinfection is particularly important if tubercle free cattle are being brought into the herd

Once a herd free of tuberculosis is established, it is, of course, essential to ensure that it is managed in such a way that reinfection does not take place Only cattle which have originated from a tubercle free source

should be added to the herd, non-reacting animals coming from herds which are not free of infection should be isolated for some 60 days and retested before addition to the herd Cattle must be transported without coming into contact with other animals, and the vehicles in which they are carried must be properly cleaned and disinfected before use No milk should be brought to the farm for feeding to calves unless it is produced in another clean herd or has been adequately heat-treated Contact with neighbouring herds must be prevented—for example by resort to double fences These precautions cover the greatest risks, but there have been occasions when healthy cattle have become infected when udder irrigation is used as a treatment for mastitis, or at the time of uterine treatment for infertility It is obvious that introduction of infection by these means into a healthy herd is bound to have disastrous consequences, and it emphasizes the need for the greatest care in the observation of personal disinfection, and the sterilization of equipment, when dealing with both infected and healthy herds Infection has also been known to be introduced into the skin of the neck from a contaminated bottle, needle, or syringe at the time of injection of tuberculin, it is thus imperative to use sterilized needles, and syringes that are kept exclusively for tuberculin testing

Vaccination with BCG has been under investigation for a number of years Whatever may be its value in man—and it would seem to have considerable value—there is no similarity between its application to the human and to the bovine subject It can be used only in calves, and they must be shown by test to be tuberculin-negative before injection, in fact in recent trials it has been demonstrated that it is important not only that the individual calf is tuberculin-negative but also that its immediate contacts have a similar tuberculin status There is little doubt that BCG confers a degree of resistance on calves in cattle, however, if a negative tuberculin test is the ultimate aim, vaccination must confer an immunity sufficient to prevent establishment of infection and not merely to limit the progress of disease if it becomes established Vaccination sensitizes the animal to tuberculin, which obviously makes its use in combination with a system of eradication depending on the test extremely difficult Vaccine might be used to protect young stock graduating to a heavily infected adult herd, but the most heavily infected herds, in general, are " flying " herds rearing very few young stock, while the most heavily infected herds which do rear calves are prone to contain calves infected from tuberculous milk at such an early age that vaccination has little practical value Equally, the fact that calves must be very carefully segregated from infection before and immediately after vaccination makes it easy to depend on segregation alone for the protection of young stock In practice, the majority of farmers whose herds were included in a recent trial proceeded to clear the

herds of infection by removal of reactors rather than by accepting the disturbance of vaccination and revaccination of their stock, particularly as, in some cases, deaths had occurred and a degree of stunting had arisen which could be attributed only to vaccination *Vaccination, even if proved* to be safe and reasonably efficient, has no place in the control and eradication of bovine tuberculosis

Annex I

THE SINGLE INTRADERMAL COMPARATIVE TUBERCULIN TEST IN CATTLE

From the Ministry of Agriculture and Fisheries London

The tuberculin test in Great Britain is applied by veterinary surgeons The tuberculin, the testing technique and the method of assessing the result are standardized in all tests applied under the State scheme

The tuberculin used is purified protein derivative (PPD) manufactured at a State laboratory and the tuberculo protein content is constant

Intradermal injections of 0 1 ml of avian and mammalian tuberculins are made in two satisfactorily spaced clipped sites on the neck after caliper measurements have been taken of a fold of normal skin After a period of 72 hours the measurements and character of the swelling at the place of injection are carefully recorded and the result is assessed as follows on the comparative sensitivity to the two tuberculins

Swellings under 3 mm negative
Swellings between 3 mm and 4 mm doubtful
Swellings over 4 mm positive

This test is intended to be applied mainly as a herd test, the interpretation of the

Britain

(1) *Evidence of non specific infection*

It has been found that where non specific' infection (i e infection other than mammalian tuberculosis) *is present in a herd the test results may have to be interpreted* differently from those in herds where no non specific infection is noted The presence in a herd of one or more animals giving a positive reaction to avian tuberculin and a negative reaction to mammalian tuberculin (A+ M—) is accepted as establishing the existence of non specific infection Clinical evidence of Johne s disease or skin tuberculosis should also be regarded as establishing the existence of non specific infection

(2) *Bases of interpretation*

The following bases of interpretation should be adopted

(*a*) Animals showing a positive or doubtful reaction to avian tuberculin and a negative reaction to mammalian tuberculin should be *retained in the herd*

(*b*) Any animal giving a doubtful reaction to mammalian tuberculin and a negative reaction to avian tuberculin should be retested

(*c*) Animals showing a positive ... a posit ... in skin ... than the ... if non ... is not e ... specific infection

(*d*) Animals giving a positive reaction to mammalian tuberculin and a negative reaction to avian tuberculin when the increase to mammalian tuberculin does not exceed the avian increase by more than 6 mm should be either (i) retested if non specific infection is established or (ii) removed if non specific infection is not established

(*e*) Animals which give a positive reaction to mammalian tuberculin and a positive or doubtful reaction to avian tuberculin when the increase to mammalian tuberculin is 5 6 mm greater than the increase to avian tuberculin should be either (i) retested if non specific infection is established, or (ii) removed, if non-specific infection is not established

(*f*) In all tests animals showing a positive reaction to mammalian tuberculin and a positive doubtful or negative reaction to avian tuberculin should be removed when the increase to mammalian tuberculin is more than 6 mm greater than the increase to avian tuberculin

Annex 2

ERADICATION OF BOVINE TUBERCULOSIS IN THE UNITED KINGDOM OF GREAT BRITAIN AND NORTHERN IRELAND

From the Ministry of Agriculture and Fisheries, London

The main instrument of the campaign against bovine tuberculosis in Great Britain is the Attested Herds Scheme which aims at the eradication of the disease from individual herds of cattle by voluntary action The initial Scheme was introduced in 1935 A farmer wishing to have his herd attested (that is certified officially as free from tuberculosis) must produce evidence that his whole herd has passed two successive tuberculin tests A further test is then made by the Government and if there are no reactors the herd is declared an attested herd The owner of the herd must obey the Rules of the Scheme which are designed to protect the herd from the risk of reinfection from outside sources Fencing round the farm must prevent contact with neighbouring stock unless these are also attested and movement of cattle onto the farm is regulated by permit Attested herds are tested periodically free of charge to check that they remain free from infection By mid June 1950 one fifth of the total cattle in Great Britain were in attested herds To stimulate voluntary action a revised Attested Herds Scheme was introduced on 1 October 1950 This provides for payment of a bonus for six years to owners of herds joining the Scheme The bonus is intended as a contribution towards the cost of eradicating the disease from the herd Under the revised Scheme, expansion of voluntary attestation has accelerated and at the end of September 1952 there were 90 811 attested

herds in Great Britain, containing 3 571 788 cattle This represents 37 5°. of the total cattle population of Great Britain A voluntary Attested Herds Scheme similar to the British Scheme was introduced in Northern Ireland in 1949

The progress made with voluntary attestation made it possible to begin the final stage in the campaign against bovine tuberculosis on 1 October 1950 when the Government introduced the area eradication plan for tuberculosis The aim is to eradicate the disease entirely from certain areas and gradually to extend those areas to cover the whole of Great Britain Suitable areas containing a good proportion of attested cattle are selected for development as eradication areas Adequate notice of intention to declare an eradication area is given and free tuberculin testing is provided by the Government in the selected area In an eradication area all the remaining untested herds are tested compulsorily and all cattle in the area which react to the tuberculin test are compulsorily slaughtered with payment of compensation Movement of cattle into an eradication area is prohibited except under licence or on certain transit journeys No untested cattle are allowed into the area and the movement of cattle onto or off particular farms within the area may be controlled by licence Other measures to prevent the introduction or spreading of tuberculosis are also enforced When for all practical purposes tuber

Since 1 October 1950 free tuberculin testing has been carried out in two areas—in south west Scotland and south west Wales—containing about 950 000 cattle On 1 October 1952 part of these areas containing 700 000 cattle became the first eradication area to be declared under the plan

Three small island areas containing about 14 000 cattle in which all the herds were attested under the Attested Herds Scheme have been designated attested areas since 1 February 1951

ISOLATION, IDENTIFICATION, AND TYPING OF TUBERCLE BACILLI

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Preparation of Media

Lowenstein's medium with 0 75% glycerol

For the culture medium, the following mixture is made up

Monopotassium phosphate	0 4%
Magnesium sulfate	0 04%
Magnesium citrate	0 1%
Asparagin	0 6%
Glycerol (twice distilled)	2 0%
Distilled water	

A flask containing 600 ml of this solution is boiled for 2 hours, after cooling, 30 g of potato flour are added and the mixture is then boiled in a water bath under continuous and thorough stirring to complete pastiness The mixture is then cooled to about 56°C before the eggs are added, 20-22 fresh eggs with large yellow yolks are cleaned carefully, broken into a large flask, shaken vigorously, and filtered through sterile gauze [illegible] ter shaking, [illegible] medium is coagulated at 85°C for 40 minutes The following day the tubes are heated to 70°C for 30 minutes The cotton plugs are then paraffined, and the tubes are stored in a refrigerator

Besredka's medium

175 ml of egg yolk, together with 500 ml of distilled water, are shaken [illegible] becomes clear This is most easily seen by drawing some of the medium up into a 10-ml pipette The figures on the pipette should be clearly visible through the medium After this, a further 6 ml of the sodium hydroxide

solution are added The medium is then diluted with distilled water to a volume of 3 5 litres It can then be poured into tuberculin flasks (80 ml in each), and test tubes (18 mm in diameter), after which it is autoclaved for 30 minutes at 110°C

Isolation of Strains from Different Specimens

Sputum

The most suspect elements are picked out, and about 1 ml is placed in a centrifuge tube To the tube are added 2-3 ml of 6% sulfuric acid, and the tube is closed with a tightly fitting rubber stopper (the broader end being pushed into the tube), it is then shaken vigorously until the contents are quite viscous, and kept at room temperature for 10 minutes The tube is then filled with sterile distilled water and centrifuged for 10 minutes, after which the sediment is spread on the media After inoculation of the tubes, they are sealed with paraffin and a hole is made in the layer of paraffin by means of a fine needle, they are incubated for six weeks

Urine and stomach lavage

After centrifugation, the sediment is treated in the same way as sputum

Spinal fluid, ascites fluid, and pleural exudate (with addition of citrate to prevent coagulation)

After centrifugation, cultures are planted from the non homogenized sediment into two tubes The rest of the sediment is treated in the same way as sputum (homogenization for only a few minutes)

Tissue, lymph glands, and clots

Cut into small pieces, and treat with 4% sodium hydroxide for about half an hour Then centrifuge, to the sediment add 1-2 drops of 8% hydrochloric acid before it is transferred to the culture media

As a rule, we use five culture tubes for each specimen—three tubes with [illegible] in order to

[illegible] numerous tubercle bacilli, the sediment for the cultures is inoculated on the surface of the medium in five tubes, as follows one loopful of the sediment is transferred to the "condensation water" of the first tube, then one loopful of this suspension is transferred to the condensation water of the next tube, and so on [illegible] form of diluted planting of the sediment is [illegible] two [illegible] comprising three culture tubes

Typing

The cultures are inspected every week [illegible] is made of all those [illegible] typ[illegible]

[illegible] xed infections are so [illegible]

[illegible] n type [illegible] result from reckoning them as belonging [illegible]

The strains of which the type cannot be decided from the appearance of the primary culture are homogenized with 4% sodium hydroxide for 20 minutes and transferred to the media, with gradations in the planting as described on page 36 in order to obtain solitary colonies ("secondary cultures") in some of the tubes We make secondary cultures in the following cases (1) when the primary growth is dysgonic, (2) when the first culture shows only a few colonies, (3) when growth is limited to the condensation water or to the glycerol-free medium, (4) when the media are infected with bacteria other than tubercle bacilli, and (5) in cases where only the inoculation of guinea-pigs gives a positive result

When using sulfuric acid alone for the homogenization, we found that acid fast and alcohol fast saprophytes might quite frequently be cultivated from the gastric lavage In the primary cultures, these saprophytes often resemble tubercle bacilli to such an extent that errors in their identification are unavoidable, even by the experienced examiner

To guard against this source of error, secondary cultures should be made, and it is recommended that some tubes with Besredka's medium (containing 5 ml of 2% malachite-green dilution per litre) be inoculated This medium is well suited to separating acid fast saprophytes from tubercle bacilli The acid-fast saprophytes cause the medium to become turbid and strongly green in colour If animals are to be used for inoculation of the culture, the Besredka culture can be transferred to a tuberculin flask containing the Besredka medium, in which the tubercle bacilli grow very rapidly, 4-7 days later, the culture can be used for inoculation of the animals This medium is particularly suitable for the production of large amounts of culture of the bovine type

The secondary cultures are typed after the same principles as the primary cultures If the growth is typically human, the strain is entered as such As mentioned, all the dysgonic strains are subcultured because it has been found that several dysgonic human strains develop into the eugonic form after one transfer In this way, the rabbit experiment may be omitted, at any rate for a good many of the dysgonic strains

There are some dysgonic human strains, however, which do not dissociate to the eugonic form, but keep on growing in such a dysgonic manner that it takes very great experience to be able to differentiate them from the bovine type merely by the appearance of the colonies Hence, it is advisable to inoculate into rabbits all strains with continual dysgonic growth

For inoculation of rabbits, we use the intravenous method, injecting 0 01 mg of culture As a rule, the rabbit dies of acute generalized tuberculosis within 30 days after the inoculation

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LABORATORY EXAMINATION OF MILK FOR THE PRESENCE OF MYCOBACTERIUM TUBERCULOSIS BOVIS

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Microscopic examination of milk samples

The traditional method of preparing films from milk deposit and staining by the Ziehl Neelsen method is used, and our experience is that the large majority of positive single cow cases can be diagnosed by this method When attention is paid to detail few errors in diagnosis are made. We examine the cellular picture closely, since this speeds up the detection of acid-fast bacilli which are often concentrated in groups of endothelial cells in tuberculous milks (see fig 1) The method is to examine the film by low-power microscope for cell groups and, when these are found, to focus them under the oil-immersion lens

We are doubtful of the significance of acid fast bacilli in milks which do not contain endothelial cells, and biological tests are carried out on these samples We find that a fairly large number of milk samples contain diphtheroids which are not decolorized in some areas of the film Their disposition in large numbers in a few areas of the slide, the fact that they are not associated with endothelial cells, and their marked pleomorphism distinguish them from *Mycobacterium tuberculosis* (see fig 2)

Mastitis resembling tuberculosis, caused by rapidly growing acid fast bacilli

Recently a small number of cases (15) of mastitis which are indistinguishable clinically from tuberculosis, but which are caused by rapidly-growing acid-fast bacilli, have been encountered The organisms are slightly larger and more pleomorphic than *M tuberculosis*, but these differences are often slight (see fig 3) Some endothelial cells may be present, but there is apparently no tendency for the organisms to be concentrated in cell groups The cut surface of affected udders is indistinguishable from that of a tuberculous udder, but no lesions are found in the supramammary lymph-nodes Microscopically the lesions in the udder resemble those of tuberculosis They are granulomas containing giant cells (fig 4), but in their centre there is a vacuole (fig 5) which contains a globule of

oil or fat (fig 6) and in which the acid fast bacilli are concentrated (fig 7, 8) Biological tests in guinea pigs, rabbits, and chickens are negative, and the condition can be set up *in cattle only when cultures* of the organisms are injected into the udder in oily suspensions All the cases diagnosed had a history of infusion of the udder with therapeutic agents in oil suspensions The affected cattle do not react to avian or mammalian tuberculins, but do react to a purified protein derivative prepared from the organisms isolated A number of different acid fast bacilli are involved, but most belong to the same group as *M smegmatis*

Biological tests

The biological test in guinea pigs is used for the final diagnosis of doubtful single cow samples, and for all herd and group bulk milk samples We use the deposit, or a mixture of cream and deposit, from 100 ml of milk for these tests From a small number of experiments we estimate that the cream and deposit of centrifuged tuberculous milk contain approximately the same number of tubercle bacilli The guinea pigs are tuberculin tested 3 weeks after inoculation and, as the correlation between the result of these tests and the postmortem findings at 6 weeks is very good, a positive result can be given at 3 weeks, when a positive tuberculin test is obtained

Culture tests

Liquid Tween 80 (a proprietary detergent) albumen, medium (Dubos[1]), is used for the primary isolation of tubercle bacilli, and in our hands it gives better results than egg media *Biological tests are, however, superior* to any culture methods for the detection of small numbers of organisms of the bovine type

Lymph nodes and organs

The macroscopic appearance of lesions and the microscopic demonstration of acid fast bacilli can generally be relied upon for a positive diagnosis of tuberculosis, except in cases of so called skin tuberculosis and lesions of the udder caused by rapidly growing acid fast bacilli Both these conditions give negative results to biological tests Biological and culture examinations are used as confirmatory tests and for typing infections We have met four cases of avian type infection of the bovine udder and, as reported also by Ottosen[2] in Denmark, we find that lesions on bovine serous membranes are sometimes caused by the avian type of *M tuberculosis* The usual site of these lesions is, of course, the mesenteric lymph nodes

1 Dubos R J & Middlebrook G (1947) *Amer Rev Tuberc* 56, 334

2 Ottosen H E (1944) *Skand VetTidskr* 34 1

FIG 1 CELL GROUP IN TUBERCULOUS MILK

FIG 2 DIPHTHEROIDS IN MILK FILM

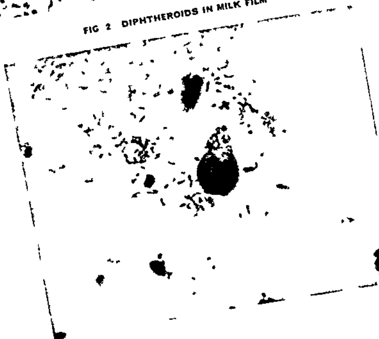


FIG 3 RAPIDLY GROWING ACID FAST BACILLI IN MILK FILM

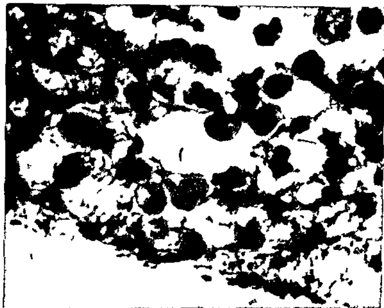


FIG 4 GRANULOMATA IN UDDER INFECTED WITH ACID FAST BACILLI — I

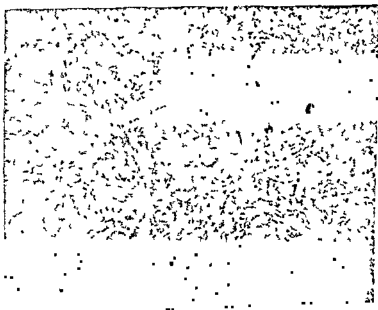


FIG 5 GRANULOMATA IN UDDER INFECTED WITH ACID FAST BACILLI — II
Central vacuole and giant cells

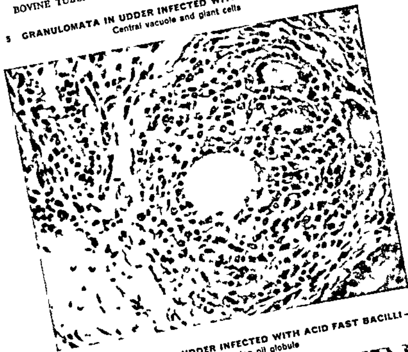


FIG 6 GRANULOMATA IN UDDER INFECTED WITH ACID FAST BACILLI — III
Vacuole showing oil globule

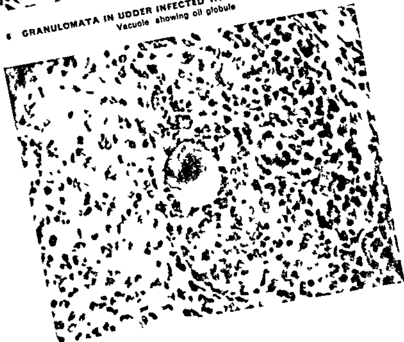


FIG. 7. GRANULOMATA IN UDDER INFECTED WITH ACID FAST BACILLI — IV
Bacilli at periphery of vacuole

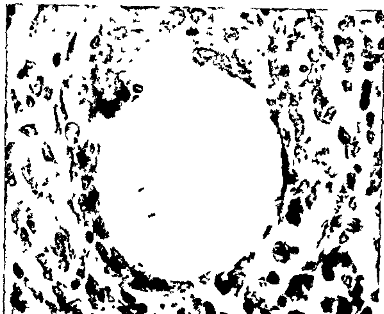
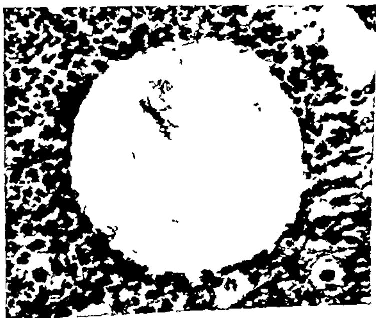


FIG 8 GRANULOMATA IN UDDER INFECTED WITH ACID FAST BACILLI — V
Vacuole with numerous bacilli



DESCRIPTION AND PREPARATION OF WEYBRIDGE PURIFIED PROTEIN DERIVATIVE TUBERCULINS

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The abbreviation PPD was coined by Seibert[1] about 1932 to denote a "purified protein derivative" prepared from the steamed concentrated bacterial filtrate of a culture of *Mycobacterium tuberculosis hominis* grown on a synthetic medium, and has since come into use for similar products derived from allied mycobacteria The word "purified" does not mean "pure" in the chemical sense, and small amounts of polysaccharide and nucleic acid are always present in any PPD, but for all practical purposes a properly prepared product represents the mixture of heat-stable tuberculoproteins which constitutes the active principle of "Old Tuberculin" (OT)

When tubercle bacilli are grown on a suitable medium, water-soluble protein, not coagulated by heat, appears in solution mainly as the result of enzymic autolysis of dying cells in the later stages of incubation If the medium is protein free to begin with, all the protein in solution is tuberculoprotein When the culture flasks are steamed to kill the bacilli, any heat coagulable protein (which may be present in small amount) is rendered insoluble and is entangled with the bacterial mass, so that on filtration the clear fluid contains the degraded heat-stable soluble protein which accounts for the specific activity of any tuberculin Although this protein, or mixture of similar tuberculoproteins of low molecular weight, behaves as an antigen in serological reactions, it does not, in small quantity, confer the skin sensitivity produced by the bacilli themselves, so that it does not induce the allergic state shown by the "tuberculin reaction" which it elicits on intradermal injection The same animal can therefore be tested repeatedly at suitable intervals

If the clear steamed filtrate is concentrated by evaporation in open vessels on a steam-bath in a current of warm air, a product of OT type is obtained, which contains the unwanted residual medium constituents, and by products of bacterial growth in concentrated form If the medium is a synthetic one a "synthetic medium OT" is obtained, in distinction to "Koch's OT" prepared from a glycerol broth medium

[1] Seibert F B (1941) *Amer Rev Tuberc* 44 1

In the process of evaporation an insoluble surface skin is formed by oxidative denaturation of part of the protein, so that more than half the active principle may be lost, and the potency of the final tuberculin is an uncertain compromise between concentration and wastage If the concentration of the filtrate is conducted in a partial vacuum, i e, at a lower temperature in the absence of air, there is very little loss of protein and a tuberculin of much higher potency can be obtained It still, however, contains unwanted medium constituents and by-products of bacterial growth which may sometimes influence undesirably the dimensions and character of the reaction produced on intradermal injection

If, however, the "tuberculoprotein active principle" is precipitated from the clear filtrate, by any suitable protein precipitant which can easily be removed afterwards, it can be separated from the unwanted residual medium constituents, redissolved at any desired strength, and issued on the satisfactory scientific basis of "a known weight of active principle in a known volume of tuberculin"

The simplest and most satisfactory protein precipitant is trichloracetic acid It precipitates protein from a medium filtrate quantitatively, so that there are no losses of active principle and the maximum possible amount is recovered from each culture flask with consequent economy in production cost, it is much cheaper "per unit of potency" to manufacture PPD than it is to manufacture OT by evaporation in open pans Trichloracetic acid is soluble in water and loosely bound to the protein precipitated by it, so that excess precipitant is easily washed away Trichloracetic acid is also soluble in ether, so that if it is desired to convert the wet protein precipitate into a dry powder, for permanent storage in a desiccator, it is sufficient to stir it up several times with anhydrous ether after centrifugal washing, and allow the ether to volatilize, leaving a physically suitable fluffy residue A preliminary washing with acetone—which, unlike ether, mixes with water in all proportions—is useful for initial dehydration of the precipitate The combined result of acetone washing, followed by stirring with ether, is to remove any readily soluble lipoidal components of the original protein precipitate, and to provide a 'dry PPD" which, for all practical purposes, contains only small quantities of contaminant bacterial polysaccharide and nucleic acid, neither of which compounds either contributes to or interferes with the intradermal tuberculin test They can largely be removed by re-solution and successive re-precipitation in various ways, and a still purer PPD obtained, but for manufacturing purposes the additional trouble and expense are not justified

In common practice the wet protein precipitate obtained from the clear filtrate by trichloracetic acid is simply washed aqueously to remove unwanted constituents of the growth medium, and re dissolved in alkaline

buffer to obtain a concentrated solution which, after addition of a preservative, can be kept in cold storage for a long time (several years) without alteration of potency The protein nitrogen of this is determined accurately by analysis, and tuberculin of any desired issue strength is prepared by dilution as required

Mammalian PPD Tuberculin

Cultivation of the bacilli is carried out by the method originally used by the Bureau of Animal Industry (BAI) in the United States of America for the preparation of their " heat concentrated synthetic medium tuberculin " The strains of organism and the synthetic medium are the same

Strains used

Human strains designated PN, DT, and C, are used, all produce good yields of protein derivative of similar characteristics The reason for using human strains instead of bovine in veterinary work is partly traditional, partly that higher yields of active principle are obtained, and partly that there is not yet sufficient evidence of a specific superiority of bovine PPD to justify a change in world practice

Reserve cultures

Four cultures of each of the three strains are kept on glycerol agar slopes, and subinoculated once a month to maintain fresh growth A stock of suitably dried organisms is also kept in reserve

Propagation medium

The medium employed is a conventional glycerol beef-broth of pH 7 3, as used by the BAI for " seeding flasks " The reason for using this instead of the synthetic medium upon which the cultures are finally grown is that the surface film on the latter is too firm for easy breaking-up into small pellicles for the inoculation of further flasks

Three cultures of each of the three strains are maintained on 100 ml of medium in 300-ml Erlenmeyer flasks and subinoculated every 10-14 days to ensure a continuous stock of rapidly-growing seeding pellicle From these a larger supply of seeding flasks is prepared, according to the number and size of the flasks used for subsequent growth on synthetic medium

Size of synthetic-medium flask

It has been found convenient to use flasks of 2-litre capacity and to charge each with one litre of synthetic medium to provide a suitable ratio of depth to diameter They are flat circular flasks with adequately strong

In current Weybridge practice a "three point assay" is used, i e, three dilutions of standard are compared with three dilutions of the PPD tuberculin to be tested Convenient dilutions for good reactions are 1/200, 1/800, and 1/3,200 The 0 2 ml injections of each dilution are randomized over the six injection sites so as to avoid bias, and reaction diameters are measured after 24 hours After rearrangement of the random readings in known order, the total reactions to each dilution are compared with the corresponding totals for the standard When these are plotted on graph paper, parallel straight lines are obtained which coincide if potencies are equal Statistical analysis of the data gives the fiducial limits of the test

A test is also made on at least six cattle in a known tuberculous herd, comparing 0 1-ml injections on each side of the neck, of strengths 1/1 (undiluted), 1/10, and 1/100, against similar dilutions of the standard preparation, and measuring the swellings by the usual technique Measurement of increase in thickness of the skin fold takes the place of measurement of diameter of erythema in guinea pigs

The fiducial limits of such a small test are, of course, not entirely accurate, and if accuracy of comparison is required the technique of multiple injections into the flanks of artificially sensitized calves is adopted

Avian PPD Tuberculin

The strain of *M tuberculosis avium* is designated D4, and has been selected from a variety of avian strains as providing a good yield of PPD of high potency per unit of protein nitrogen Although most established laboratory human strains of tubercle bacilli yield PPD of similar potency and characteristics, the potency of PPD from avian strains is much more variable, and, when issuing an avian PPD on the basis of weight of active principle per unit volumes, it is necessary to keep to a strain of which the characteristics have been well studied

Preparation procedure is the same as for mammalian PPD, except for obvious differences arising from the lower yield of tuberculoprotein derived from steamed culture filtrates Whereas human PN, DT, and C strains provide filtrates containing about 0 8–1 0 mg per ml, avian strain D4 (and most avian strains) provides filtrates containing only 0 25–0 30 mg per ml Since a good avian tuberculin of OT type, prepared by growth on synthetic medium with subsequent evaporation of steamed filtrate, and formerly used for detection of tuberculosis in birds, contains only about a quarter of the weight of active principle as does a human OT, the issue-strength of avian PPD tuberculin is decided on the basis of 0 5 mg per ml Any desired strength at all could of co

from the protein precipitated from steamed filtrate by trichloracetic acid, but the proportions of 2 0 mg per ml for mammalian PPD and of 0 5 mg per ml for avian PPD retain the traditional link with the past, and conform to the field experience upon which the English " single intradermal comparative cattle test " was originally based

The synthetic medium used for growth of D4 avian tuberculin is the same as for mammalian tuberculin The propagation medium is the same glycerol broth, and subculturing follows the same interval of 10-14 days The protein precipitate is handled in the same way, and the concentrated solution for storage is again based upon one tenth of the initial volume It is noteworthy that very few debris of high molecular weight are thrown out during passage through the Sharples centrifuge The concentrate is analysed for tuberculoprotein nitrogen, and the level for subsequent dilution to issue strength of 0 5 mg per ml is calculated from the figure obtained

Potency of routine issue material is checked against a " dry powder avian PPD " made in 1942 from D4 strain, and retained as the provisional Weybridge standard The guinea-pigs are sensitized by infection with a virulent live strain of *M tuberculosis avium* or with ground dead D4 organisms in oil (vacuum-dried bacillary debris from steamed filtrate) The dilutions used for the 0 2-ml injections are lower, to correspond with the lower tuberculoprotein content of avian tuberculin and the different degree of sensitization acquired by the test animals, but a " three point assay " is again used Suitable dilutions are 1/100, 1/400, and 1/1,600 (or 1/50, 1/200, and 1/800) Routine cattle tests are not carried out, but potency comparisons may be made on calves artificially sensitized with avian bacilli

International Standards

The PPD S of Seibert, of which 0 00002 mg tuberculoprotein content corresponds to one International Unit (IU), corresponding fairly closely in potency with the IU for OT, is the existing standard for " mammalian PPD tuberculin "

There is as yet no international unit for avian tuberculin, but a large stock of PPD from D4, in dry powder form, has now been prepared and will be offered for international use, together with protocols on specificity and viable cultures of the organism itself in suitable dehydrated form, so that any country wishing to reproduce " Weybridge avian PPD tuberculin " can do so

It is hoped that stocks of standard preparations of bovine PPD, and of tuberculoproteins from other mycobacteria of interest, will become available at a later date

Small-Scale Preparation of PPD Tuberculins

The technique of preparation at Weybridge[4] is adapted for large scale production because of the fact that about 1,000 litres (10 million doses) of each type are used per annum by the Veterinary Inspectorate of the Ministry of Agriculture and Fisheries, but smaller scale production can be carried out using 100 ml quantities of synthetic medium in 300-ml Erlenmeyer flasks, and omitting the passage of concentrate through a Sharples centrifuge Most of the debris of higher molecular weight in the tuberculin after dilution are held back by bacterial filters, clogging them and involving higher wastage of active principle, but if this is allowed for, and the final product adjusted to 2 mg of protein per ml for mammalian, and 0 5 mg per ml for avian, tuberculins, the resulting PPD tuberculins do not materially differ from one another in biological tests

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[4] *Green H H (1946) Vet J 102 267*

DISCUSSION: PART I

Several speakers pointed out that bovine tuberculosis seems to be more serious in man than has been generally believed, in some countries, it represents as much as 10% or more of all human tuberculosis Apart from its transmission through the consumption of raw milk, a route which has often been overlooked is the respiratory tract in cases where there is association with infected cattle Children on farms are frequently infected in this manner Direct contact infection also occurs in 15 individuals (all adults) studied in Denmark, primary bovine tuberculosis infection of the conjunctiva is believed to have derived from exposure to tuberculous cattle

The importance of continuously carrying out the typing of strains was stressed Some studies in Denmark have indicated the interesting fact that bovine tuberculosis strains in human pulmonary cases may undergo a gradual transformation towards the human type, although the process may take years It is very difficult to prove this beyond doubt, but prolonged studies on individual cases support the view

On the question of media for typing bacilli, the discussion leader was of the opinion that Löwenstein's medium (plus 0 75% glycerol) gave a clearer picture of growth than Petragnani's medium In some investigations where both human and bovine type bacilli were suspected in the same specimen, inoculations were made on Besredka's medium followed by inoculation into rabbits if Löwenstein's medium alone were used, it would be possible that only the human type would be found The importance and necessity of inoculating laboratory animals in connexion with the routine typing of bacilli was pointed out

Emphasis was placed on the compulsory pasteurization of all milk and milk products as the first line of defence for the general population The laborious tests on mixed milk samples for the presence of tubercle bacilli, of limited value at best, could be avoided if periodic tuberculin testing were utilized, and compulsory pasteurization required, for milk from positive herds As eradication programmes progressed there would be less and less need for this type of milk test

Attention was called to the common fallacy of considering goats as resistant to tuberculosis In Switzerland it has been conclusively shown that goats can be affected with the open pulmonary type of tuberculosis.

Work in Switzerland on the application of the haemagglutination test in cattle was reported A method developed by Professor G Schmid (Berne) has been fairly valuable in confirming tuberculin tests In bovines a titre of 1/20 is considered negative A rise in titre to 1/40, or higher, indicates infection In many cases where the tuberculin test has given doubtful or negative results, tuberculosis has been revealed by using this method An increase in the haemagglutination titre is accepted as an indication of a poor prognosis The antibodies in the haemagglutination test, it was pointed out, are quite different from those influencing the tuberculin test, positive reactors to which generally show a rather high titre to the haemagglutination test More work on this test is, however, required

The question arose of a simple method for differential diagnosis between tuberculosis strains and saprophytic strains According to Dr B Babudieri, the latter could be distinguished from the former by adding *p*-aminosalicyclic acid to the medium, tuberculosis strains were unable to multiply, whereas the saprophytes were not affected

In many countries the marking of reactor cattle is compulsory, the main argument being that there should be no risk of the animals' being sold for purposes other than slaughter In the United Kingdom of Great Britain and Northern Ireland this system has not been undertaken There no animal may be admitted into tuberculosis free herds or areas without a certificate stating it to be non tuberculous So long as the campaign was based on a voluntary scheme, it had not been found justifiable to mark the reactors while other possibly infected animals from untested herds could freely be brought into the market All clinical cases from attested herds and all reactors from tuberculosis free areas are compulsorily slaughtered, with subsequent payment of compensation

The question of false tuberculin reactors in cattle was raised The problem appears to give most trouble in places where the disease has been markedly reduced, and it is one of the major difficulties in the final steps of eradication Cases of avian and human tuberculosis in cattle give positive tuberculin reactions Avian type infection can generally be differentiated by use of the comparative tuberculin test Reactions in cattle caused by human type bacilli often disappear after several months

The advantages and disadvantages of cattle vaccination were discussed The vaccine most widely used is BCG In France it was felt to have some limited usefulness when employed under careful supervision, especially in valuable herds where a large number of animals were already infected and where the farmer could not afford to dispose of them Here, the vaccination of uninfected calves gave them some degree of protection and prolonged the useful life of the animal It was felt, further,

that since the positive reaction which results from vaccination disappears after a time, it would not interfere with the tuberculin test if eradication were ultimately attempted In Austria a killed vaccine adsorbed onto aluminium hydroxide was being tried, but it was too early to claim definite results

The disadvantages of vaccination in cattle were stressed The employment of such a weapon would postpone eradication of the disease, which should be the ultimate goal The main disadvantages indicated were interference with the tuberculin test, the difficulty of obtaining uninfected calves in highly infected herds and of avoiding exposure during the post-vaccinal period, the apparent necessity for repeated vaccination, and the undesirable reactions produced in inoculated animals It was considered that these disadvantages had resulted in the discarding of vaccination as a possible aid to control in the United Kingdom, and that far greater strides had been made by keeping to the policy of gradual eradication The bases for a successful eradication programme were again pointed out—namely, government co-operation for slaughter of infected animals, premiums on milk from non-infected herds, and co operation of the farmers, achieved through an intensive and continuous educational campaign The financial and moral assistance of public-health authorities might also be enlisted

frequent symptoms in *Br abortus* infection[3] and *Br melitensis* infection[4] show that they are similar in the two cases (asthenia, fever, anorexia, etc) Mortality seems to be greater in *Br melitensis* infection This type of *Brucella* infection is also more frequently concerned with bone or joint lesions We think that systematic examination of bone lesions, especially of the vertebral column, should *form part of the routine clinical examina* tions of patients infected with brucellosis, these lesions are frequent but as *a rule are cured if detected at* an early stage of the disease Some workers claim, on a limited number of examinations, that there are differences in the microscopic appearance of the lesions In point of fact, a comparative study of the clinical aspects and of the degree of severity of the disease according to the infective agent has still to be made before more than an impression can be given

Therapy of Human Brucellosis

For an exact estimation of the value of curative treatment of brucellosis we must always keep in mind two points

(*a*) human brucellosis is a self-curing disease in the majority of cases,

(*b*) *late* relapses, after a *long* period of apyrexia, are *frequent*

These two points should always be borne in mind when treating a *Brucella* infected person if the above points had not been overlooked, many publications would not have been printed and this matter would have been simplified From the available medical publications we can *conclude*

1 Rest in bed and an adequate diet are essential to proper treatment (It should be remembered that in practice brucellosis is the *only febrile* disease apart from tuberculosis with which a sick person can lead quite a normal life despite having a high temperature)

2 The antibiotics at present available, with the exception of penicillin, which is completely [illegible] progress in the treat *ment of the disease* [illegible] but they do not pre[illegible] little or no value, streptomycin or dihydrostreptomycin *alone is* [illegible] value Either antibiotic in combination with other antibiotics or with sulfonamide compounds appears to be active, but the toxicity, and particu larly the damage caused to the eighth nerve, should not be underestimated

Aureomycin is a valuable drug, the generally accepted schedule for adults is 2 g *daily for 14 21 days* Oxytetracycline[5] (same dosage as for

[3] Dalrymple Champneys W (1951) In Banks S ed *Modern practice in infectious fevers* London p 408

[4] Taylor R M Lisbonne M Vidal L F & Hazemann R H (1938) *Bull Hlth Org L o N* 7, 503

[5] Oxytetracycline is the non proprietary name for *Terramycin*

aureomycin) gives a similar result Chloramphenicol is also a valuable antibiotic against brucellosis, but care must be taken on account of the danger of serious aplastic anaemia following its use Simultaneous use of two or more of the drugs mentioned above has been recommended It seems that streptomycin or dihydrostreptomycin plus aureomycin or oxytetracycline gives better results than a combination of streptomycin and sulfadiazine

The most commonly accepted dosage is either 1-2 g of streptomycin or dihydrostreptomycin plus 4-6 g of sulfadiazine for 14-21 days, or the same dosage of streptomycin or dihydrostreptomycin plus 2 g of aureomycin or oxytetracycline

We think it necessary during antibiotic therapy to complete the treatment with vitamin B complex to avoid accidents from avitaminosis

Any relapses should be treated in the same way as before

3 No agreement has yet been reached on the value of antigen therapy in brucellosis—a method that has been in use for a long time—owing to the fact that the bacteriological qualities of the strains used are seldom well defined thus only rarely can it be said that smooth preparations have been used Some authors claim good results, others very poor results The best method seems to be by intravenous injection Precautions should be taken concerning possible shock phenomena

This treatment using the whole brucellae or extracts thereof, could probably be useful where antibiotics are not easily obtainable

In any case such treatment is only justified in proven cases of brucellosis Prolonged treatment is not recommended for patients complaining of various and ill defined symptoms and in whom bacteriological proof of brucellosis has not been found

Recent observations indicate that a combination of small doses of cortisone (500 mg in 2 days) with antibiotics has given promising results

Economic Importance of Brucellosis

Everybody agrees upon the economic importance of brucellosis, but data for the evaluation of losses due to this disease are seldom available This is because of the difficulties encountered in carrying out investigations It is very difficult to know in the area under investigation the rate of infection among the herds of all animal species, the number of abortions in these animals, and the losses in milk products resulting both from abortions and from the disease itself It is even more difficult (physicians do not always report the disease, although it is notifiable) to know the number of human cases and their duration, not to mention the possible complications at a later date

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2 The antibiotics at present available, with the exception of penicillin which is completely inactive, have resulted in great progress in the treatment of the disease They produce a rapid fall to normal in the temperature but they do not prevent relapses Sulfonamide compounds *alone* are of little or no value, streptomycin or dihydrostreptomycin alone *is of no* value Either antibiotic in combination with other antibiotics or with sulfonamide compounds appears to be active, but the *toxicity, and* particularly the damage caused to the eighth nerve, should not be underestimated

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Even if the number of human cases were known, it would still be necessary to determine the cost of treatment and the number of hours of work lost

Calculation of losses due to brucellosis in goats and sheep are made according to the sale value of the animals Such an evaluation does not take into account the real value of these animals for the owner, particularly in the case of goats In most cases, the goat flocks are practically the sole means of livelihood of the owner, they are also the only available animal protein source, and naturally the true value of these animals is greater than the market value

Furthermore, even if it were possible by some method to calculate the losses in animal protein (milk and meat) due to brucellosis, how could we evaluate the effects of the resulting nutritional imbalance on man's health, such as metabolic troubles, and increased sensitivity to other diseases (e g, tuberculosis or malaria), particularly in underdeveloped regions where breeding of goats and sheep is widespread

Finally, for the maintenance of the herd and for milk and meat production, the farmer needs to have a larger flock than would be necessary were it not for brucellosis This causes waste of food and of land which could otherwise be used for purposes other than grazing or forage production How may the losses to agriculture resulting from the misuse of food resources be evaluated?

For the above reasons the few estimates already published are mostly tentative, and the figures given are largely approximate and much lower than the true figures

Thus, from an inquiry based on definite data only—namely, the number of aborted farm animals and the amount of social insurance paid for the hospitalization of sick people—we estimate that brucellosis would cost more than 37,000,000 000 French francs per year in France

Control of Human Brucellosis

The way to control the spread of *Brucella* to man is clear from the above considerations We must try, on the one hand, to prevent the sick animal from infecting man and, on the other hand, to eliminate the disease in animals There is no really effective human-vaccination procedure, only hygienic measures can be applied

Individual prophylaxis

Several means may be employed to prevent human infection from sick animals All are consequent upon the application of propaganda and the education of individuals in the correct observance of rules of hygiene, which are more easily enumerated than applied Farmers and, more

generally, people in contact with farm animals should wear working clothes which are different from their everyday clothing.

This is perhaps rather theoretical, but it is true that a great number of cases of human brucellosis could be avoided if people wore gloves, washed their hands carefully after handling animals and manure, and disinfected and changed their shoes after work. At the present time, these conditions are by no means met in most farms or factories engaged in milk or meat production. An educational effort sponsored by hygienists and veterinarians would be fruitful and would considerably reduce the number of human cases by contact. Besides legislation governing the sterilization of milk or milk products for human consumption, propaganda in the rural centres is badly needed to explain clearly and in great detail the aim and importance of these sanitary measures. Here also, the role of veterinarians, as well as of hygienists is of importance: their advice and example has more chance of being followed because of their closer contact with animal breeders. Without propaganda legislation would be worthless.

General prophylaxis

Man is a sort of cul-de-sac in the brucellosis story. The *Brucella* is essentially an organism which attacks animals: one must therefore control the animal disease and this is a veterinary problem. Methods such as vaccination, and serodiagnosis followed by slaughter or segregation, have been described elsewhere. However it should be remembered that nothing justifies the vaccination of infected animals.

In order to obtain quick and accurate results, close co-operation should be maintained between the veterinary, medical, and hygiene services. Such a collaboration could result in:

(1) Utilization of common specialized laboratories for diagnosis of human as well as animal brucellosis. Such a combination would be of some advantage in:

(*a*) saving of money; (*b*) providing well trained technicians, and (*c*) facilitating the use of correct and accurate techniques in conformity with the standards defined in the reports of the Joint FAO/WHO Expert Committee on Brucellosis.

Furthermore such a centralization of diagnostic methods would afford quicker action in brucellosis control, for example, by indicating to the veterinary authorities the possible focus shown by one new human case.

For correct epidemiological study in areas where brucellosis is prevalent, this joint action is indispensable. We can affirm having found and treated many human cases through epidemiological studies carried out in close co-operation with the veterinary services.

A central laboratory common to all those engaged in the control of brucellosis, would, by automatically collecting results, greatly facilitate the acquisition of more exact knowledge concerning the extent of the disease Such a laboratory would also permit the extension of research on brucellosis and *Brucella*

(2) Common agreement upon regulations on the detection and control of brucellosis

(*a*) general recognition of brucellosis as an occupational disease,

(*b*) *hygiene of establishments concerned with the production of meat* and milk,

(*c*) regulations on the obligatory *sterilization (pasteurization) of milk* and other milk products,

(*d*) notification of human and animal cases,

(*e*) regulations on the systematic examination and surveillance of flocks

It would also be good to arouse the personal interest of peasants by

(1) Establishing certified areas of farms In such places the owners would accept periodic survey by the veterinary services In turn, the owners would be authorized to sell their milk or meat at an increased price if the animals were found healthy In such a manner, human cases of infection by ingestion or contact would probably be more quickly reduced than by the use of " force "

(2) Declaring that brucellosis, like tuberculosis, is a latent defect that makes a sale void, this would prevent the less scrupulous farmer *from selling infected animals*

(3) Requesting the presentation of a certificate indicating that the serodiagnostic test is negative in all animals exhibited at a cattle show, thus the reputation of the owner would be at stake

(4) Inducing breeders to produce healthy animals by prohibiting the introduction into a given country or area of positive reacting animals

We again insist upon the great importance of propaganda relative to the control of brucellosis An educational programme is more conducive to success than regulations Such propaganda must be made by a joint effort, with close liaison between the *Ministries of Health and of Agriculture*, and employing all technical resources available, such as lectures, *press notes, films*, and broadcasts An intensive propaganda campaign would encourage " free prophylaxis " which would be as efficacious as prophylaxis by authoritative measures

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ANIMAL BRUCELLOSIS

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The control or eradication of brucellosis is a necessity both on economic grounds and on those of human health. In animals, the disease is associated with a high abortion- or prematurity-rate and consequent reduction of milk yield, and infertility is often a sequel. In humans, it causes a great deal of illness and inability to work, especially in areas or in occupations where conditions are conducive to transmission of infection from animals to man and where those infections are with *Brucella melitensis* or *Brucella suis*.

Prevalence

Realistic estimates of the prevalence of brucellosis are difficult because, in most countries, it is not yet a notifiable disease in man or animals.

Br. abortus infection in animals reaches 10% to 20% or 30% in many European countries and similar figures have been reported from certain countries of South America. In the United States of America, the latest figures indicate that about 4% of cows are infected and 16% of herds. Infection was present to a similar or less extent in the Scandinavian countries but it has now been eradicated from Norway and very much reduced in Denmark and Sweden. Estimates of its prevalence in Great Britain are complicated at the present time by vaccination. However, before governmental measures were taken for its control, a survey of over 30,000 pregnancies[19] showed that 9.7% ended abnormally—5.4% in abortion and a further 4.3% with a dead calf, and from other evidence it seems likely that most of the abortions and many of the dead calves were due to *Brucella* infection. *Br. melitensis* infections in goats and sheep are known to be widespread in many countries of the Mediterranean area and in goats in Latin America, while *Br. suis* infections are common in certain areas of the USA.

The data available in regard *to infections in human beings* are also inadequate, but some figures of interest have been published by the World Health Organization in their *Epidemiological and Vital Statistics Reports* The figures for the cases reported for 1951 include the following Algeria 39, Eritrea 81, Morocco 34 Canada 189, Mexico (1950) 1,041, Peru 373, *United States of America (1950) 3,163, Iraq 21, Israel* 30, Austria 61, Belgium 25, Denmark 153, France 1,095, Federal Republic of Germany 298, Greece 181, Italy 8,924, Malta 609, Netherlands 63, Norway 1, Portugal 305, Scotland 107, Spain 4,387, Sweden 7, Switzerland 166, Turkey 29, Yugoslavia 18, Australia 34, and New Zealand 55[37] The figures were similar to those for the preceding five years, except in the case of those for Eritrea, Germany, and Greece, which had risen, and those for Norway, Sweden, and Denmark, which had dropped *markedly* A *large number* of cases are not diagnosed and reported, and it is believed, therefore, by many that the prevalence of the disease is much higher than is indicated by the above figures

Species of Brucella, their Differentiation and Distribution

The main distinguishing characters of the recognized species of *Brucella* are shown *in table I*, and *most strains—provided they are* in the *smooth* phase—show both the biochemical and serological characters of one or other of these species Strains with the biochemical characters of *abortus* and the serological characters of *melitensis* have, however, been reported from France, India, Great Britain, and the USA, while a considerable proportion of strains isolated in Italy and Tunis, and some from the USA, have the biochemical characters of *melitensis* but the serological characters of *abortus* There are also a smaller number of strains which are atypical biochemically in one respect or another, or which have more or less equal amounts of *abortus* and *melitensis* antigens

One eminent worker[29, 30] takes the view that the differences in the species generally recognized are insufficient to warrant the retention of species differentiation, but whether they be regarded as *differences of species* or variety, all workers are agreed on their reality and practical value provided serological tests are applied only to strains in the smooth state and certain well known precautions are taken in regard to other tests

All species can occur in human beings and in most of the domesticated [illegible]

and also in cattle, in *Mediterranean countries, in goats in* [illegible] and in cattle and pigs in the USA, *Br suis* occurs in pigs in the USA and

TABLE I DIFFERENTIAL CHARACTERS OF BRUCELLA SPECIES

	Br abortus *	*Br melitensis* *	*Br suis* *
Aerobic growth	−	+	+
Hydrogen sulfide production	++	− or trace	++++ †
Growth in presence of			
thionin	−	+	+
basic fuchsin	+	+	−
methyl violet	+	+	−
pyronin	+	+	−
Agglutination in monospecific sera for			
Br abortus	++++	−	++++
Br melitensis	−	++++	−

* The number of + signs indicates approximately
(a) for hydrogen sulfide production the amount and duration
(b) for dyes the relative amounts of growth
(c) for agglutination the number of tubes showing agglutination in an ordinary doubling series

† Strains isolated in Denmark did not produce hydrogen sulfide

has also been reported from Denmark and central Europe *Br melitensis* and *Br suis*, in that order, cause the most serious disease in human beings, but a curious anomaly exists in that strains isolated in Great Britain, which are typical of *Br melitensis* in every other respect, have been isolated from a considerable number of cows in recent years without any evidence of illness among consumers of infected milk from these animals, although it is known that much has been drunk in the raw state.

It has long been known that cattle can be infected by ingestion, by way of the conjunctiva, by way of the skin, broken or unbroken, and also per vaginam, as well as by injection, and it is known that guinea pigs can also become infected by inhalation of quite small numbers of brucellae It seems likely that infection may occur in most of these ways in other animals and in human beings The actual route in any species will depend largely on the kind of exposure

In animals, the origin of infection is usually an aborted foetus, or infected genital discharges, or urine, and it seems quite likely (though it has never been proved) that infection can occur during the process of milking

In human beings, infection by ingestion plays a prominent part, the main source of danger being raw cream and milk and certain products made from these, such as some soft cheeses Living *Brucella* has also been found in the viscera, flesh, and lymph-nodes of infected carcasses

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for periods of more than a month after death of the animal, and it is known that *Brucella* can survive pickling, but not commercial smoking, though it does not seem likely that these are common sources of infection Contamination of water supplies, such as wells, by infected animal excreta has also been incriminated, as has the use in salads of greens contaminated with sheep or goat urine and faeces Contact with infected animals, such as contact with a foetus or placenta, or with urine, manure, or an infected carcass, is a common cause of infection in exposed people—for example, slaughterhouse workers, animal attendants, farmers, veterinarians, and in fact anyone who comes into close contact with animals or their products In some countries, contact infection is believed to account for 40%-70% of cases It is also believed that infection may be dust- or droplet borne and infect via the eye or respiratory tract Accidental inoculation has also occurred in the laboratory and in the field Methods of prevention of human infection include personal hygiene, environmental sanitation, and heat treatment of suspect foodstuffs

In human beings, the incidence of infection in the young and in the adult female is much lower than in adult males, and it seems likely that this is due to an actually greater degree of resistance, because—although the peak age for male infections is also one during which exposure would be highest—there are reports which make it clear that in some areas the stable is virtually a part of the house and the women and children must be as exposed to infection as the adult males

There seems to be no doubt that the young bovine is more resistant than the adult Nothing can be said about sex rates in animals because of the large proportion of males which are killed early in life and the lower degree of infection to which those which are reared are usually exposed

Control of Brucellosis in Bovines

Various methods of control have been adopted in different countries but they all fall under three headings those based on the elimination of infected animals detected by diagnostic tests, those based on vaccination and those in which the two methods are combined

The serological test has nearly always been the agglutination test in the past, but in recent years the milk ring test has come into use, particularly as a screening test

Regional differences in the prevalence of infection and in economic and educational status make it necessary that the methods used should be chosen to suit the country, area, or herds concerned It has been amply demonstrated that the disease can be eradicated from herds, areas, or

countries solely by the elimination of reactors to diagnostic tests It has also been shown that the clinical effects of the disease can be overcome by appropriate vaccination, and that the incidence of infection can be considerably reduced In herds or areas where the disease is acute and the incidence of new infections is high, vaccination may be the method of choice or the only method practicable in the first place. Where, however, the incidence of infection is relatively low, the disease should be eradicated by test and elimination of reactors or by combination of this with vaccination of the young animal in such a way that it does not interfere with the use of diagnostic tests

Whatever method is used, it is of the first importance that proper hygienic and sanitary precautions should be taken to prevent spread of infection from such things as aborted foetuses, placentas, genital discharges, and materials which may become contaminated therewith The spread of infection from males particularly in the early stages of infection, is another important danger to be borne in mind

A more detailed discussion of various of the points concerned will be found in the report on the first session of the Joint FAO/WHO Expert Panel on Brucellosis [36] It is there urged that countries which have at present no active programme for the control and prevention of brucellosis should initiate such programmes as soon as practicable It is suggested in that report that countries which have not yet adequate services or laws, rules, and regulations should take measures for the enactment of such laws, for the appointment of professionally trained personnel of permanent status to operate the regulations, for the operation of general control and preventive measures of such a kind as to prevent further infection being brought into the country or distributed, for vaccination with strain 19 (for a discussion of this, see section entitled "Vaccination" page 77), and for inauguration of an educational programme of such a kind that it will reach all segments of the population and include the training of veterinarians and technicians The last-mentioned measure must in many countries be undertaken as a primary necessity

Countries with adequate personnel and laws are, moreover, urged to take more active steps, including the control of distribution of vaccine, the permanent identification of reactors, and the control of movement of reactor and untested cattle

It is recommended that in areas with a low prevalence of infection (10% or less of the herds infected, and 3% or less of the individual animals infected) the main methods should be test with elimination of reactors, with or without vaccination of heifer calves in infected herds, together with test and temporary retention of reactors, with vaccination of heifer calves and possibly of mature negative animals in certain herds where it is necessary

In areas with a moderate to high prevalence of brucellosis (10% 35% of the herds infected, and 3% 10% of the individual animals), the policy recommended is similar, except that more latitude is necessary in regard to vaccination of adults in highly infected herds, and that vaccination of heifer calves so situated or managed as to be exposed to infection is regarded as desirable

In areas or countries where brucellosis reaches about 35% of herds or 10% of total animals, and where there is shortage of trained control personnel vaccination without test may be the only practicable measure

Methods of Diagnosis

Tube serum agglutination test

This is generally regarded as the most practicable serological test *Wide variation has existed in the past in the methods and antigens used* for tests of both man and animals [32] and steps were taken many years ago by the Office International des Epizooties (OIE) to standardize the interpretation of the test for bovines The OIE standard dried serum was also taken as an international basis for interpretation of the test in human beings, goats, and swine by the Joint FAO/WHO Expert Committee on Brucellosis at its second session,"[8] and the WHO Expert Committee on Biological Standardization [33] has since established an International Standard for Anti *Brucella abortus* Serum equivalent in potency or titre to the OIE standard The titres recommended as positive for bovines are now defined in terms of this standard serum as follows "the minimum positive titre should be between 1/10 and 1/12 of the titre obtained when the OIE standard serum is tested with the antigen and by the methods of the country concerned ' [34] (p 20) Criteria recommended for interpretation of the test in man, goats, and swine will be dealt with elsewhere

Rapid or plate test

This is regarded as an adequate substitute for the tube test, but it is usually recommended that it should be adjusted to correspond It has an advantage over the tube test in that sera which fail to react or show *prozones in* the tube test, owing to blocking antibodies or for other reasons, commonly react to the plate test

Complement fixation test

This provides results which are usually comparable with those of the ... agreed

error

Milk ring test

This is proving itself a valuable screening test to locate infected herds, especially in areas of low infection where much blood testing can thus be avoided It is used to a considerable extent in Denmark, Sweden, and the USA The test is essentially one for agglutinating antibodies in milk, which clump the stained bacteria present in the antigen, which are then brought up by the fat globules to produce a coloured cream ring It depends, therefore essentially on the amount of antibodies in the milk, but it is influenced greatly by the proportion of cream and by a number of other factors The test is very sensitive and is positive when the milk of an infected cow is mixed with that of several others Further work is needed on the criteria which can be taken as indicative of infection in individual cows Vaccination with strain 19 in calfhood or up to the age of 18 months does not interfere with the test in any important way, provided an interval of 6 months or longer elapses between vaccination and test More information is required regarding the effect of adult vaccination on the ring test The antigen can be stained with haematoxylin or with tetrazolium chloride

Plate test with stained antigen

This test is particularly suited for milk examination and gives fewer false positive results than the ring test The ring test antigen is suitable

Other diagnostic methods in bovines include bacteriological and biological examinations of foetal or placental material or of milk and microscopic or complement fixation tests with placental material The intradermal test has also been used

Vaccination

The ideal vaccine would be one which confers a sufficient degree of protection which is safe because it is dead or relatively avirulent and has no tendency to increase in virulence in the animal, which causes a minimum of interference with diagnostic tests and finally which is not too costly or difficult to produce keep and distribute

Living vaccines of high or unknown virulence have been used in the past but they may be the means of spreading infection and should not be used

Huddleson[14, 15] in the USA in the 1920 s was the first to suggest that attenuated strains might resolve the problem of control, but unfortunately the strains with which he worked did not confer sufficient resistance Cotton[6] and Cotton Buck & Smith[7, 8] a little later examined a number

of strains of low virulence and selected one, the now well known strain 19, because of its high immunizing power and low virulence Buck[3] showed further that animals vaccinated at the age of 6 9 months usually lost their agglutinating antibodies by the time of their first parturition, but retained an adequate immunity This method of vaccination, therefore, fits well with eradication projects and has been made use of in the USA Denmark, and Sweden in particular

Work was carried out also on other attenuated strains in Great Britain by McEwen[21 24] with his strain 45/20, but though this gave a satisfactory immunity, it was also unfortunately shown that the strain could revert to the virulent form Other strains have been examined, particularly in Denmark and Germany, but none has proved better than strain 19

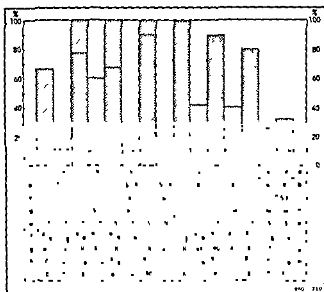
Dead vaccines were used a great deal in the past Dead vaccines in saline are of no value dead vaccines in oily excipients can, however, confer considerable protection Gilbert,[12] for example made a number of investigations with a dead vaccine composed of virulent brucellae killed with formalin and suspended in an oily vehicle Experiments with different doses, vehicles, and sites of injection showed that a dose of 500 000 to 600,000 million organisms in 5 ml of 10% lanolin in liquid paraffin conferred a high degree of immunity against a dose of *Br abortus* which caused most unvaccinated animals to abort[31] The vaccine is dead and therefore safe, and it can also be stored The large number of bacteria required make it, however, more costly than a living vaccine such as strain 19, while it confers a less adequate protection against large challenge doses Some *Brucella* extracts and ether killed vaccines with adjuvants have given promising results in experimental animals, but further work is needed

Huddleson[16] also prepared a vaccine containing *Brucella* in the mucoid phase, but preparation is difficult, and cattle experiments have not so far shown it to be of equal value to strain 19

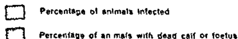
Much experimental work in cattle has been done with strain 19 In the USA, Mohler, Wight & O Rear[28] summarized a number of the experiments with strain 19 carried out by Cotton Buck & Smith Of 70 vaccinated animals only 8 (11%) became infected after an exposure which caused infection in 57 out of 73 controls (78%), and of these 55 aborted or had premature calves Thomsen[34] in Denmark tested strain 19 vaccine in six herds, in which two thirds of the animals were vaccinated and one third left as controls Of 266 vaccinated animals, 3 3% aborted, while of 135 controls, 25 1% aborted

Results obtained in Great Britain to date with various of the vaccines mentioned above are shown side by side in fig 1 and 2 Those made with killed vaccines, marked K in the figures, were carried out at the Weybridge veterinary laboratory All of the remainder were made at the

FIG 1. EXPERIMENTS IN BOVINES WITH BRUCELLA ABORTUS VACCINES



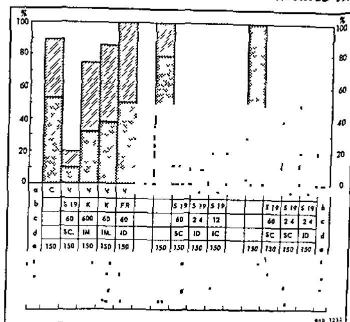
(a) Control (C) or vaccinated (V)
(b) Vaccine (45/20 = Strain 45/20 S 19 = Strain 19 K = killed vaccine)
(c) Dose (organisms × 10⁹)
(d) Injection route (SC = subcutaneous IM = intramuscular)
(e) Challenge dose (organisms × 10⁶)
(f) Proportion of animals infected
(g) Percentage of animals infected
(h) Proportion of animals with dead calf or foetus
(i) Percentage of animals with dead calf or foetus



Field Station of the Agricultural Research Council of Great Britain at Compton, Berks, England [10, 11 19 20]

In all these experiments, except the last, the animals were vaccinated about two months before service, and the challenge dose was given when the animals were about five months pregnant This challenge dose was always prepared with the same strain (*Br abortus* 544) which is kept dried at Weybridge and has been used for all tests of immunity in guinea-pigs, goats, or cattle since the strain was first titrated by McEwen et al in 1939 [25] Except for the first experiment, in which a dose of 1 5 million organisms proved too small, the experiments were made with a challenge

FIG 2 EXPERIMENTS IN BOVINES WITH BRUCELLA ABORTUS VACCINES



(a) Control (C) or vaccinated (V)
(b) Vaccine (S 19 = Strain 19 K = killed vaccine FR = *Brucella* fraction)
(c) Dose (organisms × 10⁹)
(d) Injection route (SC = subcutaneous IM = intramuscular, ID = intradermal, IC = intracaudal)
(e) Challenge dose (organisms × 10⁶)
(f) *Proportion of animals infected*
(g) Percentage of animals infected
(h) Proportion of animals with dead calf or foetus
(i) Percentage of animals with dead calf or foetus

 Percentage of animals infected

 Percentage of animals with dead calf or foetus

dose of 15 millions, or for later experiments 150 millions, and the results in the controls were fairly consistent The vaccine used in the earliest experiment—45/20 (fig 1)—produced a satisfactory degree of resistance, but as stated earlier it mutated[33] and is no longer used. Killed vaccine protected well against a test dose of 15 millions (fig 1), but, even in a dose ten times that of strain *19*, failed when challenged with a dose of 150 millions (fig 2) The *Brucella* fraction used in these experiments showed little or no increased resistance against the large test dose. Strain 19 conferred a high level of protection in all except the last experiment

Route of vaccination

The last two experiments in fig 2 are concerned with route of vaccination. In the penultimate experiment, it will be seen that equal and adequate protection was given by the usual 60,000 million dose subcutaneously, by one fifth of this dose intracaudally into the dense tissue under the tail, and by intradermal injection of one twenty fifth of the subcutaneous dose It was thought that the subcutaneous dose itself may have been unnecessarily large, and the last experiment, which has not yet been published, was intended to test this point Unfortunately, no group showed the degree of protection found in earlier experiments and, though no difference is seen, the results are not satisfying The reason is not known, although it is to be noted that, contrary to usual practice, the animals in this last experiment were vaccinated shortly after service The check tests which are made in guinea pigs with all vaccines used for big experiments gave protection results which were well up in the characteristic range and did not suggest a deficiency of the vaccine

Experiments made in the USA (C A Manthei—personal communication) have also shown that reduced dosage by the intradermal or subcutaneous route may protect for the succeeding pregnancy, while Gregory [13] found in comparable groups of about 40 heifers vaccinated at 7 10 months of age that 1 ml intracaudally (?12 000 million organisms) was at least as effective as 5 ml subcutaneously More information about the minimal dose to give satisfactory protection is, however, needed, especially in regard to its effect on duration of protection, and the Joint FAO/WHO Expert Committee on Brucellosis at its second session (1952) [48] took the view that no reduction in the dose of 60,000 million viable organisms could yet be recommended

Duration of resistance conferred by strain 19

The information regarding the duration of immunity either for animals vaccinated as calves or for those vaccinated later in life is not yet adequate What reports there are suggest that immunity may already be diminished a little by the second calving [9] but that it is adequate for most natural exposures Some American workers have reported a low degree of protection by the third [2] or the fourth pregnancy,[2] but in other experiments [25] there was no decrease, a finding in which the authors believe increased age immunity may have played a part Field results have shown no reduction in immunity in later pregnancies,[6, 17, 35] but the exposure was probably decreasing year by year as abortions were brought under control and infection was correspondingly reduced

Further extensive experiments are in progress in the USA and in Great Britain (at Compton) to find out more about the duration of immunity,

and in Great Britain attempts are also being made to determine the relative value of repeated administration of vaccine at different ages

Stability and safety of strain 19

There can be no certainty that a living vaccine will not change, but strain 19 has so far proved remarkably stable Vaccines must, however, be composed of organisms in the right growth phase and have their proper content of viable organisms when injected

Passage experiments in the bovine,[27 33] in which a normal heifer was made to abort by the intravenous injection of a very large dose and in which the *Brucella* obtained from the foetus was passaged by the intra venous route successively through six or in the British case,[33] seven further animals, failed to show any change in virulence There is no evidence that animals vaccinated in the normal way excrete the organisms or that strain 19 is spread from vaccinated to unvaccinated animals Transient excretion has occurred in animals vaccinated while pregnant, but no human cases have been traced to this origin It should be remembered, however, that human infections can occur from accidental inoculation with strain 19 and that care should therefore be taken by those handling it

Reactions following injection

A definite temperature reaction follows injection of strain 19 in animals, and they should therefore be housed for a short time during cold weather There may also be some local swelling, particularly if care is not taken to distribute the vaccine at the site of injection In the adult, there is also a definite, though usually temporary, loss of milk yield More severe reactions are sometimes encountered

Serological reaction

In the calf, as in the adult, agglutinins appear a few days after injection and attain their maximum after about 28 days Their titres can then be similar to those of natural infection In calves of 6 8 months, these titres are less than those of older animals, they soon diminish and the majority have disappeared within six months A few positive reactions remain at the time of the first calving The nearer the animal is to puberty when the vaccine is given, the longer does the agglutination titre remain above the normal These persistent agglutination reactions are of no practical importance in themselves, but are a serious disadvantage where vaccination is being combined with an elimination of infected animals on the basis of blood tests, because there is no way of distinguishing them from those caused by natural infection with virulent strains

Strain 19 is an extremely valuable vaccine and nearly satisfies the criteria set out earlier, but continued research on the subject is desirable

Results of Various Control Methods in Different Countries

Elimination of reactors to the agglutination test and sanitary measures have been the sole or main basis of control in a number of countries, notably Norway, Denmark, Sweden, the USA, Canada, and Finland, while the method has been used in individual herds in many other countries and in large areas in Germany and Palestine

Norway

A scheme for the eradication of the disease was introduced in 1934 Aborting animals had to be reported and tested, and the owner of infected animals was required to see that his animals did not have a chance of infecting those of others by sale, service, or contact of any kind whether on the home farm or on the common pastures Infected animals were marked and could only leave the farm for slaughter In 1936 there were 2,758 infected herds The disease has now been eradicated Some setbacks were met, but it is stated that only some 2%-3% of herds accepted as ' clean", " relapsed' or became reinfected

Denmark

Great headway has also been made in Denmark, the percentage of herds free from infection in 1946, 1947, 1948, 1949, and 1950 being 32 9, 67 3, 77 3, 86 9, and 89 6 respectively Cases of human brucellosis during the last five years have totalled 0 7 per 10,000 inhabitants, or about half the rate for the 1931-40 period The milk ring test is now used widely for diagnostic purposes Calf vaccination with strain 19 vaccine is combined with elimination of infected animals

Sweden

In 1938, 5 5% of Swedish cattle were infected In July 1951, the corresponding figure was 0 4% Reported cases of undulant fever have dropped from 114 in 1939 to 16 in 1950

United States of America

The Federal project for the eradication of bovine contagious abortion was begun in 1934 when it was estimated that 10% of cattle were infected Some setbacks were met with in certain herds and areas, and new outbreaks of the disease occurred in 2%-5% of the herds believed to be free In 1949 however, tests on over five million animals showed 4% to be positive to the agglutination test, while the corresponding figure for 1950 was 3 5% The milk ring test is also used widely in the USA as an ancillary method of diagnosis, particularly in certain States and in areas which are relatively free from brucellosis

Vaccination with strain 19 is combined with elimination methods, and three methods are officially approved (*a*) test and slaughter of reactors, (*b*) test and slaughter, with calf vaccination, (for both these methods indemnity is paid for slaughtered animals), and (*c*) test and conservation of reactors, with calf vaccination Vaccination of the whole herd has become more favoured, particularly in certain areas and in certain kinds of herd The agglutination reaction which follows the vaccination of adults interferes, however, with the subsequent employment of the agglutination test and interferes, therefore, with its use for the elimination of reactors Official opinion in the USA still has as its goal the complete eradication of this disease

Finland

A good deal of progress was made in Finland before the second World War, but a serious setback resulted from the uncontrolled movement of animals during the war period Progress is now again being made

Great Britain

Preliminary investigations carried out in Great Britain in the early thirties showed that the disease could be eradicated from individual herds, but emphasized also the dangers of reinfection of a "clean" herd At the beginning of the second World War, however, it was decided that for the time being it would be necessary for various reasons to adopt a policy of vaccination The general results have been satisfactory in that, although we do not know the percentage of herds still infected, brucellosis is no longer an important economic problem[17] It is hoped in the future to eliminate the disease

New Zealand

Strain 19 vaccine has also been used widely in New Zealand and with very satisfactory results[4, 5]

Vaccination is also the main method of control in certain other countries, although exact data are not available

Control of Brucellosis in Other Animals

Brucellosis in goats and sheep

Infected goats are a dangerous source of brucellosis, especially since the disease in these animals often assumes a chronic form without obvious symptoms Immediate steps to eliminate the infection are obviously

desirable in any newly infected area and elsewhere where practicable, but unfortunately in many of the endemic areas these desiderata are extremely difficult to carry out Sanitary measures to prevent spread of infection from animal to animal and from animal to man are therefore of the greatest importance, but, again, may not be easy to put into practice Wherever possible, goats reacting to diagnostic tests should be slaughtered, but if this is impossible, all efforts should be made to evolve satisfactory sanitary and isolation measures, including destruction of infected corrals, disinfection of infected material, and segregation of infected animals and herds [a]

Brucellosis in sheep, as opposed to the disease in goats, is often self-limiting, and slaughter does not appear to be essential as it is in the case of goats Efforts to control the disease by segregation of reacting sheep can therefore be used with more confidence

Vaccination with strain 19 has been tried in goats and sheep but is reported to be of no value Some encouraging results have been obtained with certain other vaccines and must be followed up in view of the importance of caprine and ovine brucellosis, particularly from the human point of view Large experiments are already under way under the aegis of FAO and WHO respectively

Brucellosis in pigs

Vaccination with strain 19 has not proved satisfactory in pigs, and although a vaccine made of *Br suis* of reduced virulence has given good results in swine, little is known of its stability Control depends therefore on segregation and elimination of infected animals This is complicated by the fact that, while the agglutination test is effective as a herd test, it is less so in individuals Fortunately, brucellosis in swine is usually self-limiting, particularly in females Disposal of the whole herd is recommended where practicable An alternative plan, which has proved practicable is to regard the whole herd as an infected unit, but to use the non-infected animals to provide clean offspring which are weaned and tested at 8 weeks of age and reared in isolation to form a new herd Criteria for use of the sero agglutination test recommended by the Joint FAO/WHO Expert Committee on Brucellosis are similar to those for goats

In areas where swine brucellosis occurs, it is an important economic problem and a public-health hazard

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To sum up concerning animal brucellosis, I cannot do better than quote the report of the Joint FAO/WHO Expert Panel on Brucellosis on its first session

"Conditions existing in the different countries of the world vary so much that the universal use of any one technique or procedure for solving the brucellosis problem is impossible The panel realizes that it will be very difficult to inaugurate active programmes in some areas and countries, but the panel believes very strongly that such control measures as can be put into practice should be adopted immediately. The complete elimination of brucellosis from herds, cattle, and countries should be the final goal" [36] (p 16)

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LABORATORY METHODS FOR DETECTION OF BRUCELLOSIS

Ministry of Agriculture and Fisheries Veterinary Laboratory, Weybridge Surrey, England

Diagnosis of Brucella abortus from Suspect Foetus and Milk

Examination of foetus

The foetus and placenta are collected as soon as possible

Films made from the cotyledons and stained by a modified Ziehl-Neelsen method are examined microscopically It is quite often possible to detect *Brucella*, stained by the fuchsin and showing as clumps against the blue background

The following organs are removed with sterile precautions ground with sand and saline, and used for cultural and biological tests spleen, lung, and stomach contents When the foetus is badly contaminated the brain is also taken

Cultures are made in Petri dishes containing a suitable dye-medium and then incubated in an atmosphere of 10% carbon dioxide for 7-10 days In addition, Petri dishes containing blood agar are inoculated to assist in the detection of other bacteria These are identified in the usual way

A bulk sample from all the organs is mixed in a mortar and inoculated intramuscularly into two guinea pigs After six weeks the guinea pigs are killed and the spleen cultured as above Agglutination tests are carried out on samples of blood collected at death

Suspicious colonies obtained from cultures are picked off and sub-cultured for subsequent identification A Gram positive non motile organism giving the typical growth and colony appearance of *Brucella* and agglutinating in a known *Brucella* serum but not in normal serum is presumably *Brucella*

Examination of milk

Clean milk samples are collected in suitable bottles If the samples have to come from a distance, boric acid is added to give a final con centration of 0 1% They are centrifuged at 1 000 revolutions per minute

(r p m) for 15 minutes This should separate the cream in a distinct layer on the top of the milk The milk from beneath the cream is poured off into a test tube, and three or four drops of cheese-making rennet are added to every 10 ml Coagulation of the casein takes place, and the water clear whey settles out This may be hastened by incubating at 37°C for half an hour Agglutination tests are made with the whey

The cream and sediment left in the bottle are mixed and cultured on dye medium in 10% carbon dioxide as already described Suspicious colonies are picked off and subcultured for subsequent identification The remaining cream and sediment is inoculated intramuscularly into two guinea pigs (1 ml each) After a period of six weeks these are killed, an agglutination test being carried out on the blood and the spleen being cultured

Medium

Ordinary nutrient agar, pH 7 5 melted and cooled to 50°C add 5% normal serum, 1% dextrose, malachite green 1/500,000, and gentian violet 1/250,000, and pour into Petri dishes

The dyes vary and the dilution to be used is checked before each new batch of dye is used

Modified Ziehl Neelsen method for microscopic detection of Brucella abortus

Carbol fuchsin (10%) for 3 minutes
Acetic acid (0 5%) for not more than 30 seconds
Wash in water for 1 3 minutes
Methylene blue (1%) for 30 seconds

Differential Tests for Brucella abortus, Br melitensis, and Br suis

Two methods can be used

1 A typical colony can be transferred from the original isolation plate or tube to a large agar-slope and the growth on this, after 2 3 days' incubation, used to inoculate differential media

2 Three large agar slopes can be inoculated from a typical colony on the original isolation plate (or tube), one being incubated in air and two in 10% carbon dioxide, one of the latter is used for hydrogen sulfide production and, subsequently, for the preparation of a suspension for serological test with monospecific sera, the other for sowing a culture for stock purposes and for preparing suspensions for circulating dye media

Method 2 is the routine Weybridge method, but method 1 is used when no isolated colony of sufficient size is available When any doubt

whatever exists, serial subcultures are made with the initial isolate to ensure purity

Carbon-dioxide requirements

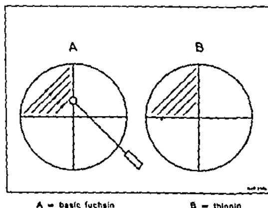
Br melitensis and *Br suis* grow under ordinary atmospheric conditions, even in primary culture from infected material, but *Br abortus* needs added CO_2 (10% is used) Most *Br abortus* strains soon lose this dependence on increased CO_2 tension, and tests should therefore be made at isolation or as soon as possible afterwards (see 1 and 2 above) The plug should not be flamed because this produces enough CO_2 to enable some *Br. abortus* strains to grow if the plugs are tight or the tube sealed

Occasionally, a freshly isolated strain of *Br abortus* will produce one or two isolated colonies when incubated in air, but the CO_2 needs of different strains provide one of the more useful tests Aerobic strains with the other characters of *Br abortus* may be derived from aerobic vaccine strains

Inhibition by dyes[1]

To a quantity of serum-dextrose agar at 50°C is added basic fuchsin sufficient to give a concentration of 1/25,000, and to another quantity of agar, sufficient thionin to give a concentration of 1/50,000, these concentrations having been predetermined with well tested type strains From each sample, Petri plates are prepared and allowed to cool. Table I shows growth in presence of methyl violet, but this is not normally used

FIG 1 USE OF LOOP FOR INOCULATING DYE-PLATES WITH BRUCELLA STRAINS



[1] Huddleson I F (1943) *Brucellosis in man and animals* New York

Suspensions are prepared from the cultures to be tested, together with suspensions of known strains of *Br abortus*, *Br melitensis*, and *Br suis* (about 3,000 million brucellae per ml viable count)

Dye plates prepared as indicated above are marked off in quarters and each quarter is inoculated with a different strain known or under test Five strokes are made on each quarter, commencing near the edge with a loopful of inoculum and progressing without recharging the loop so that the one nearest the centre receives a very small inoculum (see fig 1)

We prefer this method because five inocula of different sizes, from each strain, are compared on the same plate, the first stroke being thick and the last very thin This overcomes the difficulty that unsatisfactory results may be obtained if too small or too large an inoculum is used

The plates are incubated in an atmosphere of 10% CO_2 for five days (see table I)

Culture material for inoculating dye plates for identification purposes should always be grown on media without dyes Cultures grown even one generation on media containing dye may be profoundly modified Cultures should not be in the rough phase

Hydrogen sulfide production

Slopes of serum dextrose agar are inoculated with the suspected and known strains of *Brucella* respectively Into each tube is inserted a strip of filter-paper previously prepared with lead acetate Incubate as above and examine every 24 hours, replacing the lead acetate paper each day if it should become blackened

TABLE I COMPARISON OF BRUCELLA CHARACTERS

Species	Carbon dioxide requirement	Hydrogen sulfide production: day 1	2	3	4	5	Cultural growth in presence of: basic fuchsin 1/25 000	thionin 1/50 000	methyl violet 1/50 000	Serological monospecific serum: *Brucella abortus*	*Brucella melitensis*
Brucella abortus	+	+	+	+	−	−	+	−	+	+	−
Brucella melitensis	−	−	−	−	−	−	+	+	+	−	+
Brucella suis *	−	++	++	++	+	+	−	+	−	+	−

* Some strains of *Br suis* (Danish type) do not produce hydrogen sulfide some (like *Br abortu* produce only a moderate amount

Serological tests

Suspensions of suspected and known strains of *Brucella* are prepared and diluted to approximately tube 4 of Brown's opacity tubes (about 6,000 million organisms per ml) A series of agglutination tests using monospecific *abortus* serum and monospecific *melitensis* serum and the above antigens are prepared and incubated at 37°C for 20 hours (see table II)

TABLE II SEROLOGICAL CHARACTERS OF BRUCELLA

Suspension	Monospecific *Brucella abortus* serum				Monospecific *Brucella melitensis* serum			
	1/20	1/40	1/80	1/160	1/20	1/40	1/80	1/160
Brucella abortus	+++	+++	+++	+++	—	—	—	—
Brucella melitensis	—	—	—	—	+++	+++	+++	+++
Suspected strain — 1*	+++	+++	+++	+++	—	—	—	—
Suspected strain — 2*	—	—	—	—	+++	+++	+++	+++

* It will be noted that the first suspected strain has the serological characters of *Br abortus* while the second has those of *Br melitensis*

Freshly isolated strains are usually in the smooth growth phase, but if atypical results are obtained they should be critically examined for dissociation In the case of older strains steps should be taken to ensure they are smooth, especially for serological differentiation

Preparation of lead-acetate strips for detection of hydrogen sulfide

Soak filter paper in 10% lead acetate and allow to dry overnight in the incubator Cut into strips 8 cm 0 5 cm, sterilize in a test tube, dry in the oven, and use as desired

Preparation of Br abortus and Br. melitensis Monospecific Antiserum

For the large scale production of these sera, bovines quite negative to the *Brucella* agglutination test may be used When smaller volumes (up to 500 ml) are needed rabbits are suitable

A number of large rabbits with prominent ear veins are inoculated weekly with 1 ml of a suspension of a proved smooth strain of *Br abortus* and *Br melitensis*, respectively, or bovines inoculated with 20 ml, each

suspension containing about 5,000,000 000 organisms per ml Small samples of blood are withdrawn periodically and tested for agglutination titre When a sufficiently high titre has been obtained (e g , 1/1,280) suitable quantities of blood are collected 50 75 ml from each rabbit or up to 6 litres from each bovine The serum ultimately obtained is preserved with phenol (final concentration 0 5%) Volumes of 100 ml of suspension of both *Br abortus* and *Br melitensis* containing about 15,000,000,000 organisms per ml are centrifuged at 3,000 r p m for half an hour and the supernatant fluid poured away This should leave a thick deposit of bacteria in each tube

To the *melitensis* bacteria add the *abortus* antiserum, and to the *abortus* bacteria add the *melitensis* antiserum , mix the contents of each tube until Incubate at 37°C for eight hours, ne cold store overnight Centrifuge oipette off the bacteria free serum from each tube

A series of agglutination tests is set up, using suspensions of *Br abortus* and *Br melitensis* When using sera of high titre, it is sometimes found that the bacteria have not absorbed all of their respective agglutinins from the serum This necessitates re absorption until a four tube difference is obtained with the homologous and heterologous suspensions respectively An example is given in table III, which shows the results of agglutination tests following the first attempted absorption of antibodies by their respective bacteria and following a second absorption

Acriflavine Test

This test has been used to detect colonial dissociation in strains of Brucella

A solution of acriflavine (concentration 1/1,000) is prepared and stored in a brown bottle, in which it remains stable

Large slopes of serum dextrose agar are inoculated and incubated for 72 hours The growth is washed off by the addition of 10 ml of saline and then adjusted to an opacity approximately equivalent to tube 4 of Brown's opacity tubes (6,000 million organisms per ml, viable count)

Macroscopic test

1 ml of this suspension is placed in a small agglutination tube and 0 1 ml of 1/1,000 acriflavine is added and mixed

If the culture is antigenically smooth, no change occurs and the suspension remains uniformly turbid If the culture is antigenically rough, a flocculent precipitate appears almost immediately, and if allowed to stand at room temperature for 1-2 hours deposition occurs, leaving a clear supernatant

TABLE III RESULTS OF AGGLUTINATION TESTS AFTER ABSORPTION AND RE ABSORPTION OF ANTIBODIES BY HETEROLOGOUS BRUCELLA STRAINS

Suspension	*Br abortus* antiserum absorbed with *Br melitensis*					*Br melitensis* antiserum absorbed with *Br abortus*				
	1/10	1/20	1/40	1/80	1/160	1/10	1/20	1/40	1/80	1/160
	First absorption									
Brucella melitensis	+++	+++	+	—	—	+++	+++	+++	+++	+++
Brucella abortus	+++	+++	+++	+++	+++	+++	+++	+	—	—
	Re-absorption									
Brucella melitensis	+++	—	—	—	—	+++	+++	+++	+++	+++
Brucella abortus	+++	+++	+++	+++	+++	+++	—	—	—	—

Mucoid colonies form a slimy precipitate, and intermediate colonies do not show any precipitate or may show a flocculation consisting of particles which are smaller and more granular than those obtained with rough colonies

Microscopic test

Inoculate plates containing serum-dextrose agar with the *Brucella* strains under test and incubate for 72 hours Pick off suitably sized colonies from each plate Emulsify each in a loopful (4 mm) of saline on a microscope slide to give a homogeneous suspension To each suspension add 1 drop of 1/1,000 acriflavine solution and mix with an applicator stick or loop

If the colony is antigenically smooth, the mixture remains homogeneous, and microscopic examination will show Brownian movement If the colony is antigenically rough, flocculation will result and all Brownian movement will cease

suspension containing about 5 000,000,000 organisms per ml Small samples of blood are withdrawn periodically and tested for agglutination titre When a sufficiently high titre has been obtained (e g , 1/1,280) suitable quantities of blood are collected 50 75 ml from each rabbit or up to 6 litres from each bovine The serum ultimately obtained is preserved with phenol (final concentration 0 5%) Volumes of 100 ml of suspension of both *Br abortus* and *Br melitensis* containing about 15 000 000 000 organisms per ml are centrifuged at 3 000 r p m for half an hour and the supernatant fluid poured away This should leave a thick deposit of bacteria in each tube

To the *melitensis* bacteria add the *abortus* antiserum, and to the *abortus* bacteria add the *melitensis* antiserum , mix the contents of each tube until homogeneous suspensions are obtained Incubate at 37°C for eight hours mixing well every half hour Stand in the cold store overnight Centrifuge at 3,000 r p m for 15 minutes and pipette off the bacteria free serum from each tube

A series of agglutination tests is set up, using suspensions of *Br abortus* and *Br melitensis* When using sera of high titre, it is sometimes found that the bacteria have not absorbed all of their respective agglutinins from the serum This necessitates re absorption until a four tube difference is obtained with the homologous and heterologous suspensions respectively An example is given in table III, which shows the results of agglutination tests following the first attempted absorption of antibodies by their respec tive bacteria and following a second absorption

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Macroscopic test

1 ml of this suspension is placed in a small agglutination tube and 0 1 ml of 1/1,000 acriflavine is added and mixed

giving a magnification of about 15 diameters is set in position, and a concave mirror is placed on the bench about 10 cm in front of the stage A microscope lamp which will give a concentrated beam of light is placed about 15 cm in front of the mirror and about 10 cm higher than the mirror In this manner the rays of light are reflected from the mirror upwards to the culture plate at an angle of approximately 45° (see fig 2)

The determining of colonial types depends mainly on differences in colour and opacity, and these are more readily distinguishable when the cultures are grown on glycerol dextrose agar poured in very thin layers in good quality Petri dishes Smooth phase colonies are generally smaller than non smooth variants and take on a light blue or greenish colour Rough phase colonies are more opaque, usually cream coloured and very granular in texture Many types of colonies not falling into either of these categories will be observed They belong to the intermediate group Selection of these colonies for stock cultures should be avoided as they may give rise to variant forms on subculture

Standardized Brucella abortus Agglutination Concentrate and Standardized Brucella abortus Agglutination Suspension

Preparation

Stocks of an aerobic strain of *Br abortus* (strain 99) which have been suspended in rabbit serum and freeze dried in vacuo, are kept in cold store

1 Reconstitute a dried culture by adding a few drops of broth and mixing Inoculate suitable slopes or flasks of serum dextrose agar and incubate for 72 hours at 37°C The resultant growth is washed off with 0 85% saline and used for the required number of Roux flasks

2 Prepare large Roux flasks of potato agar Inoculate each with approximately 2 ml of the seed culture and spread over the surface of the agar by rocking backwards and forwards Incubate the flasks for 72 hours at 37°C in the horizontal position agar uppermost Carefully inspect each flask and discard any which are contaminated From the remainder pour off and discard the surplus liquid which is usually found in each flask

3 From tubes which have previously been prepared and sterilized pour into each flask approximately 20 ml of normal saline containing a few glass beads Agitate the flasks till the growth is washed from the surface of the medium

4 Then separate the liquid from the beads and from any particles of agar by filtering through a funnel containing a pad of glass wool

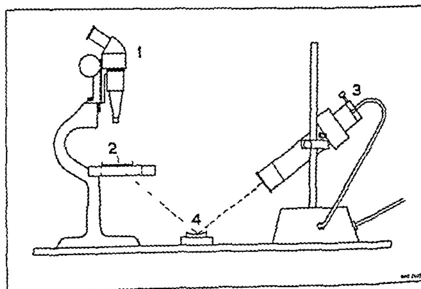
The following shows the results of microscopic acriflavine tests on the various types of colony

True smooth types S	Typical smooth reaction i e uniformly turbid
Intermediate types I	Varying results
Rough types R	Typical rough reaction i e, flocculation and if allowed to stand separation of floccules from surrounding liquid
Smooth R types S^R	Typical rough reaction
Mucoid types M	Typical mucoid reaction, i e slimy and thread-like flocculation
Smooth M types S^M	Typical mucoid reaction

Oblique-Light Technique for Observation of Bacterial Dissociation in the Brucella Group

The observation of bacterial dissociation in the *Brucella* group is accomplished by examination of plate cultures under a low power microscope *using obliquely transmitted light* The method was first described by B S Henry in 1933 [2] A binocular microscope with a lens combination

FIG 2 POSITION OF MICROSCOPE AND LAMP FOR OBLIQUE LIGHT OBSERVATION OF BACTERIAL DISSOCIATION OF BRUCELLA



1 = Microscope (magnification x15)
2 = Glass stage with culture plate
3 = Lamp giving concentrated beam of light
4 = Mirror

[2] Henry B S (1933) *J infect Dis* 52 374

a dark background The dilution of suspension selected is that which shows 2+, or 50%, agglutination at a 1/480 final dilution of the dried serum In the example given in table IV a 1/22 dilution of the bulk concentrate gives 1+ agglutination at 1/480 final serum suspension dilution and a 1/24 dilution gives 2+ at 1/480, with ± at 1/560 A 1/24 dilution of the bulk concentrate is taken This constitutes Standardized *Brucella abortus* Agglutination Suspension

4 To facilitate ease in storage and dispatch it is kept as Standardized *Brucella abortus* Agglutination Concentrate In the above example, each millilitre of the unstandardized concentrate (i e , the original bulked suspension from the flasks) is diluted to 2 4 with 0 5% phenol saline, and this constitutes Standardized *Brucella abortus* Agglutination Concentrate Before use, one volume is diluted with nine volumes of 0 5% phenol saline This is Standardized *Brucella abortus* Agglutination Suspension

Medium used for growth of bacteria

Potato agar

1 Sound raw potatoes are washed and pared and 250 g sliced thinly into 1,000 ml of water with minimum exposure to air

2 The mixture is held overnight in a covered container at approximately 60°C and is then filtered through filter paper

3 The filtrate is made up to 1,000 ml with distilled water and the following ingredients added sodium chloride—United States Pharmacopoeia (USP)—5 g Bacto peptone or equivalent—10 g , beef extract, Leibig or equivalent—5 g , dextrose (USP)—10 g , agar (USP), washed—either 20 g (for stock culture) or 30 g (for seed and antigen production)

4 The mixture is heated to dissolve agar

5 20 ml of glycerol (USP) are added and the medium is adjusted to a pH of 7 40 This results after autoclaving in a pH of 6 8 If other than distilled water is used, adjustment must be made by trial in order that the final product may have a reaction of pH 6 8 The hot solution is passed through a Buchner funnel containing two thin layers of absorbent cotton

6 The filtered medium is poured into suitable Roux flasks in sufficient quantity to make a layer 1/4-inch (6 35 mm) thick when the flask is placed in a horizontal position The flasks are plugged with non absorbent wool, autoclaved for 30 minutes at a pressure of 15 pounds per square inch (p s i) (1 kg per cm²), and then laid in a horizontal position until the medium has solidified

5 Kill the bacteria by heating for one hour at 60°C in a water bath Allow to cool and filter through a pad of cotton-wool

6 Add phenol to give a final concentration of 0 5% This concentrated suspension is kept in cold store for at least a week before standardization

Standardization

Stocks of ampoules of standard dried serum, each containing the dry matter from 1 ml of anti *abortus* serum of titre exactly equivalent to the International Standard Anti-*Brucella abortus* Serum (freeze dried in vacuo), are kept in cold store

1 An ampoule is taken and the serum reconstituted by adding 1 ml of sterile distilled water With the reconstituted serum, make dilutions in phenol saline of 1/160, 1/200, 1/240, 1/280, and 1/320 The dilutions of serum are kept in cold store ready for use but are renewed at least every three months

2 Mix the concentrated suspension well and dilute 1 ml with phenol saline until it corresponds to about tube 4 of Brown's opacity tubes, for example, 1 ml of suspension + 19 ml of saline, i e, 1/20 Make further dilutions of concentrated suspension (e g, 1/22, 1/24, 1/26, 1/28)

3 Into five series of suitable tubes (i e, one series for each dilution [illegible] concentrated suspension) place 0 5 ml of each of the dilutions of concentrated suspension, mixing [illegible] serum, i e, 1/320, 1/400, 1/480, 1/560, and 1/640 Incubate at 37°C for 20 hours and examine against

TABLE IV SERUM DILUTIONS REQUIRED IN TEST FOR CHOICE OF STANDARDIZED BRUCELLA ABORTUS AGGLUTINATION SUSPENSION

Dilution of concentrated suspension	Initial dilution of serum				
	1/160	1/200	1/240	1/280	1/320
	Final dilution of serum in serum suspension mixture				
	1/320	1/400	1/480	1/560	1/640
1/20	+++	++	±	−	−
1/22	+++	++	+	−	−
1/24	+++	+++	++	±	−
1/26	+++	+++	++	+	−
1/28	+++	+++	+++	++	+

Recording of results

Tests are incubated at 37°C for 20-24 hours and read by ordinary light against a darkish background, the results being recorded as follows.

++++ = complete agglutination and sedimentation, i e, 100% or water clear
+++ = about 75% clearing or nearly complete agglutination and sedimentation
++ = about 50% clearing and marked sedimentation
+ = about 25% clearing and distinct sedimentation

Occasionally, definite or nearly complete specific agglutination will occur with little or no sedimentation Such agglutination is to be distinguished from coarse particles, usually small in number, which occur in contaminated sera Prozones are rare with the standard suspensions but may occur in the first, or possibly first and second, dilutions with sera of very high titre

Interpretation of results [3]

No agglutination or agglutination at 1/10 but less than ++ (i e, 50%) at 1/20	Pass
++ (i e 50%) agglutination at 1/20 but less than ++ at 1/40	Indefinite (retest)
++ (i e 50%) agglutination at 1/40 or over	Fail

In case of doubt, an opacity standard for 50% agglutination should be used

Plate (or Rapid) Test for Blood Serum or Milk Whey

Preparation of test suspension

The concentration of cells in the plate suspension is so adjusted that when it is used and read in the manner described below, the results and classification of the animal shall be the same as that obtained in the tube test

Serum dextrose agar

Ordinary nutrient agar, pH 7 5, melted and cooled to 50°C Add normal serum and 1% dextrose, and allow to cool in flasks or tubes (It is essential that the serum contain no *Brucella* agglutination antibodies)

Brown's opacity tubes

Tube 4 is approximately equivalent to 6,000 million *Brucella* organisms per ml

Method of use

Successive batches of test suspension are standardized to uniform agglutinability by means of a permanent standard dried *Br abortus* serum, so that, provided the test methods are similar, all tests made with them shall be comparable, irrespective of place and time

Standardized *Brucella abortus* Agglutination Concentrate as dispatched from this laboratory should be diluted by mixing 1 volume with 9 volumes of phenol saline This produces Standardized *Brucella abortus* Agglutination Suspension This suspension is then ready for use for method 1 but should be diluted with an equal volume of phenol saline for method 2

Method 1 The method of setting up agglutination tests on serum or whey at the laboratory of the Ministry of Agriculture and Fisheries and in most other laboratories using the suspension is as follows

Serum dilutions are made by the serial method, four or five dilutions being made for diagnostic purposes (final dilutions of 1/10 to 1/80 or 1/160) or a longer series where the end titre is required, the most practicable and commonly used sizes of tube and total volumes being 1 ml in 2 inch × 3/8 or 5/16 inch (50 mm × 10 mm or 8 mm) tubes, or 2 ml in tubes about 3 inches × 1/2 inch (76 mm × 13 mm)

(*a*) *For final volume of 1 ml* 0 8 ml of 0 5% phenol saline is placed in the first tube of the series and 0 5 ml in the remainder 0 2 ml of serum is then added to the first tube, the contents mixed, and 0 5 ml of the mixture carried to the second tube The process is repeated until the last tube is reached where after mixing, 0 5 ml is discarded To these 0 5 ml volumes of serum dilutions (1/5 and upwards) is then added 0 5 ml of suspension, giving a series of final dilutions from 1/10 upwards

(*b*) *For final volume of 2 ml* The method is the same as for (*a*) except that all volumes are doubled

Method 2 Small quantities of whole serum are measured into a series of tubes and sufficient suspension, diluted with an equal volume of saline added to give final serum dilutions of 1/10 to 1/80 or 1/160, or as needed

Spreaders Wooden applicator sticks, the ends of which can be broken off after each test, thus avoiding the possibility of contaminating other tests Wire is not recommended, since it scratches the glass

Method

The lights in the box should be switched on to warm the plate just before use Both serum and suspension should be at room temperature

With the pipette at an angle of 45°, place in neighbouring squares on the plate 0 1 ml, 0 05 ml 0 025 ml, and 0 0125 ml of the serum sample Thoroughly mix the bottle of suspension, and with the dropping pipette in a vertical position place one drop (0 03 ml) of suspension at the side of each spot of serum Commencing with the highest dilution, mix thoroughly with the stick and leave a circular spot 3/4 inch (2 cm) in diameter, pass on to the next dilution, leaving spots of increasing size until a circle 1 $^{1}/_{8}$ inches (3 cm) in diameter is made with the lowest dilution All these spots should remain moist for about the same time Break off the end of the stick Lift the glass plate from the box and rotate with a circular motion till homogeneous mixtures are obtained (about 15 seconds) Replace the plate, turn off the light, and close the lid to prevent evaporation After eight minutes the tests are finally examined

About three minutes before final reading of the tests, the plate should be lifted, rotated two or three times, and replaced in the box At eight minutes the lights should be switched on, the box tilted gently so that the mixtures flow from side to side, and observations made against the dull black background

Classifications are made as follows + = complete agglutination, which is taken as the end point (it may be coarse agglutination occurring early, or fine agglutination taking eight minutes for completion), ± = agglutination occurring within eight minutes but obviously not complete, – = negative

Interpretation

0 1 ml	0 05 ml	0 025 ml	0 0125 ml	
+	+	+	+	Fail
+	+	+	±	
+	+	+	–	
+	+	±		Indefinite
+	+	–	–	
+	+	–	–	Pass
+	–	–	–	

A concentrated suspension is first obtained from growths on Roux flasks in the same way as for tube test suspension In this case, however, it is usually necessary to concentrate the suspension still further before standardizing This is done by centrifuging and re-suspending to give a concentration of approximately 120 times that of Brown tube 4, or 800,000 million organisms per ml

Standardization

To the 1-ml volumes of this concentrate are added varying volumes of phenol saline, e g , 1 ml + 0 1 ml , 1 ml + 0 2 ml , 1 ml + 0 3 ml , etc These mixtures are tested by the plate method with standard dried serum diluted 1/12 in negative serum (this negative serum must be completely negative at a dilution of 1/5 in the tube test) The mixture which shows definite but not immediate agglutination with the 0 025-ml amount of this serum dilution (the third spot in the method described below) is chosen as the correct mixture The remainder of the concentrated suspension is then correspondingly diluted, bottled, and placed in cold store It will be noted that the amount of serum (0 025 ml) is the same as that in the 1/40 dilution with the tube test, although the actual dilution of serum on the plate is quite different

The test

Although the dilutions of serum in the serum suspension mixture are quite different in the tube and plate tests respectively, the plate antigen has been concentrated and adjusted so that when one drop (0 03 ml) is mixed with volumes of serum equivalent to those which are used in the corresponding tube tests (1/10, 1/20, 1/40, 1/80), it will give similar results It should be noted, however, that, while results comparable to the tube test are usually obtained, occasionally a sample of serum is encountered which reacts differently with the plate and tube tests Such samples are uncommon

Apparatus required

Testing box This consists of a box 2 feet × 1 foot × 7 inches (approximately 60 cm × 30 cm × 18 cm), on top of which is placed a thick glass plate marked off in 1½ inch (4-cm) squares It is an advantage to have a lid covering this plate to prevent evaporation The interior of the box should be painted dull black and the lights inside arranged so that only oblique light reaches the plate

Pipettes To measure volumes 0 1 ml, 0 05 ml, 0 025 ml, and 0 0125 ml

Antigen dropper Made from a Pasteur pipette or otherwise, and adjusted to deliver 100 drops to 3 ml, i e , approximately 0 03 ml per drop

be done within one minute of adding antigen The presence of darkly stained areas in the top layer of the milk, after standing for a few minutes, indicates improper mixing

The tubes are heated for 30 45 minutes at 37°C in a water-bath and the results are read as follows

Definite blue cream ring, white milk-column	=	+++
Definite blue cream-ring, slightly blue milk-column	=	++
Definite blue cream-ring, definite blue milk-column	=	+
Cream layer only slightly bluer or same colour as milk-column	=	±
White or slightly blue cream layer, blue milk-column	=	—

The tubes used should be of a size to contain 1 ml of milk in a column 2 cm high While tubes of other sizes can be used with larger volumes of milk and antigen so as to give a milk column of similar height, this is of no advantage and is uneconomical of antigen

The antigen should be stored in a refrigerator but not frozen

Plate Tests with Whole Milk using Ring-Test Antigen

This test is carried out according to the method described by Blake, Manthei & Goode [6] This is as follows

On a glass plate place 0 08 ml of milk and add 0 03 ml of ring test antigen Thoroughly mix with an applicator stick and read after 12 minutes according to the following interpretation

+ = very slight agglutination (the amount that would be detected up to 25%)
++ = easily detected agglutination (approximately 50%)
+++ = approximately 75% agglutination
++++ = approximately 100% agglutination

The glass plate used for testing is the same as that used for the rapid method of testing of serum

Preparation of a Standard Dried Serum

An International Standard Anti *Brucella abortus* Serum has been established by the WHO Expert Committee on Biological Standardization [7]

[6] Blake G E Manthei C A & Goode E R jr (1952) *J Amer vet med Ass* 120 1

[7] World Health Organization Expert Committee on Biological Standardization (1953) *World Hlth Org techn Rep Ser* 68

Brucella Ring Test

Preparation of haematoxylin antigen[4]

Two hundred and fifty ml of a 5% aqueous solution of ammonium aluminium sulfate are mixed with 120 ml of glycerol and 135 ml of a 4% solution of haematoxylin in absolute alcohol. One gram of sodium iodate dissolved in about 15 ml of distilled water is then added. The oxidizing process is continued until the solution assumes a deep burgundy colour (5-10 minutes at room temperature), and is then broken off through dilution of the aforesaid colour stock solution with 4,750 ml of a 5% solution of ammonium aluminium sulfate. The dyeing liquid is generally prepared the day before and filtered before use. Then the pH of the filtered dyeing liquid is lowered to about 2.60 with normal hydrochloric acid, and the centrifuged *Brucella* bacteria are suspended in this solution in the proportion 1 g of bacteria to 25 ml of staining liquid. The pH is then raised to 3.0, by addition of normal sodium hydroxide, and the suspension of *Brucella* is heated to 80°-85°C for about ten minutes.

After staining, the bacterial suspension is centrifuged, and the liquid is poured off carefully. If the staining solution cannot be removed completely, the stained bacteria may be re-suspended and washed once[5] in tap-water acidified to pH 3.0 by means of hydrochloric acid.

Finally, the stained bacteria are suspended in 0.85% saline with 0.5% of phenol in the proportion of 1 g of stained bacteria to 20 ml of the liquid. This antigen is stable for several months at ice box temperatures.

If longer storage-life at ordinary temperatures is desired, the bacteria should be suspended in pure glycerol.

The sensitivity of the antigen can be adjusted by varying the density of the bacterial suspension, as the sensitivity is approximately a linear function of the degree of dilution.

The colour of the antigen can be intensified by using larger amounts of staining solution and by prolonged heating at higher temperatures during the staining process.

Method of use

To 1 ml of milk in a narrow test-tube add 1 drop of antigen (0.03 ml) and, avoiding foaming, mix gently by inverting several times. This should

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[4] Tetrazolium antigen is also used and is prepared by the method described originally by H. Bendtsen in a multigraphed paper issued by WHO as Brucellosis Information Series No. 3 (22 June 1950).

[5] Recent batches have been washed three times with an improvement in the specificity of the results.

agreement would be reached by workers making observations in different countries, and for this the 50% end point is best. It can be determined with considerable accuracy by taking the average of five series of tests with closely spaced dilutions around the expected titre, e g, if a preliminary test shows the titre to be between 1/640 and 1/1,280, the dilutions used would be 1/640, 1/800, 1/960, 1/1,120, and 1/1,280

Opacity standards for reading the degree of clearing (which may be taken as a measure of the degree of agglutination) can be prepared as follows

Stage 1 (similar for all methods of test)

Tube 1, for no agglutination	undiluted antigen
Tube 2, for 25% agglutination	3 parts antigen, 1 part saline
Tube 3, for 50% agglutination	2 parts antigen, 2 parts saline
Tube 4, for 75% agglutination	1 part antigen, 3 parts saline
Tube 5, for 100% agglutination	saline alone

Stage 2 (varies according to method)

(*a*) For the method in which antigen is added to an equal volume of serum dilution add to each tube above, an equal volume of saline[8] or of the appropriate saline dilution of a completely negative serum (i e, negative at one tenth to one-sixteenth of the titre regarded as positive)

(*b*) For methods in which antigen is added to small volumes of whole or diluted serum add to each tube above saline (or saline dilution of negative serum) equal to the volume of whole or diluted serum

(*c*) Make appropriate adjustments for other methods

For convenience, opacity standards appropriate to the method concerned, prepared as above, are spaced in a rack and the tube to be read is matched between them

Preparation of Brucella abortus (Strain 19) Vaccine

Stock cultures of strain 19, dried in rabbit's serum in vacuo, are kept in cold storage. A new tube of dried culture is used for each batch of seed material

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[8] Saline can be used when the tubes are to be used for reading high dilutions of serum (which have no colour) e g when testing the sensitivity of a method by means of the International Standard Anti *Brucella abortus* Serum. The appropriate dilution of a proved negative serum is necessary however when they are used for comparison with low dilutions of serum especially if these are deeply coloured

a 1 0-ml volume of distilled water This reconstituted serum is then made up to a 1/40 dilution in 0 5% phenol saline, the ampoule and tip being finally washed out at this stage From this, dilutions of 1/160, 1/200 1/240, 1/280, and 1/320 can readily be prepared in approximately 40-ml amounts as follows

19 ml of 1/40 dilution of serum + 57 ml of phenol saline = 1/160
8 ml of 1/40 ,, ,, + 32 ml of ,, ,, = 1/200
7 ml of 1/40 ,, ,, + 35 ml of ,, ,, = 1/240
6 ml of 1/40 ,, ,, + 36 ml of ,, ,, = 1/280
20 ml of 1/160 ,, ,, + 20 ml of ,, ,, = 1/320

When equal volumes, e g , 0 5 ml, of these serum dilutions and of the *Brucella* suspension to be tested are mixed, the final dilutions of the dried serum are 1/320, 1/400, 1/480, 1/560, and 1/640

Quintuplicate tests as here described, together with standard controls representing 75%, 50%, 25% and 0% agglutination, are incubated at 37°C for 20 hours and then compared in a suitable reading box

Replicated tests, read to a 50% agglutination end point with opacity standards prepared with the suspension being tested, are regarded as essential for accurate standardization of new test suspensions and for preparation of standard sera

50 % Agglutination in Standardization and Interpretation of Agglutination Test

The 50% agglutination end point is adopted because it is believed that this is the most suitable end point for exact assessment Lower degrees of agglutination are less easily assessed with accuracy, and "complete agglutination" is an unsatisfactory end point for two reasons (1) some workers insist on absolute crystal or water clarity (i e , 100%), while others record degrees of agglutination varying from 75% to 100% as "complete" and (2) some antigens clear completely at serum dilutions about one half of that which gives 50% agglutination (i e , they have a sharp end point), and others only clear completely at dilutions which are a quarter or an eighth of those at which 50% agglutination occurs More-over, if several workers are asked to make exact readings of a series of tests, there will be better agreement in regard to the 50% agglutination end point than to any above or below

For routine testing it will be regarded by many as more convenient to continue to use their traditional end point

For estimation of the sensitivity of a given method of test, however, it is essential to have an accurately measured end point, and one on which

11 The standardization is next carried out The bacterial strength of the vaccine should be 16 000 million viable organisms per ml It has been found by experience that in properly prepared vaccine this viable count is equivalent to a total cell content of 0 72% This may be determined by any convenient method and a dilution factor arrived at The Hopkins tube method gives a fairly accurate standard Four Hopkins vaccine tubes are used Into each tube 0 5 ml of concentrated suspension is pipetted, followed by 4 5 ml of distilled water, and the tubes are centrifuged at 2,750 r p m for 75 minutes The height of the column of packed cells is noted The average volume of packed cells in the four tubes multiplied by 200 gives the percentage of cells by volume Therefore, the percentage of cells divided by 0 72 gives the final volume to which each millilitre of concentrated suspension is to be diluted

12 Dilute the pooled suspension to the required standard in amounts of approximately 2,000 ml The diluting fluid is buffered saline, pH 6 3, and is sterilized in flasks with a hooded pipette attached The vaccine is bottled under the hooded pipettes into 5 ml (one-dose) bottles

13 1% of the final bottled product is purity-tested for seven days At the completion of the test the bottles are sealed with a plastic cap and labelled with a serial number and expiry date The latter is 28 days after the test is completed

14 Viability determination A series of dilutions, culminating in one of 1/10,000,000, is prepared in normal saline 0 1 ml of the final dilution is plated on to each of six glycerol-dextrose agar plates, spread with a sterile glass spreader and incubated at 37°C for 6 days A vaccine containing 16,000 million viable organisms per ml should allow about 160 colonies to develop on each plate

15 0 1 ml of a 1/100 dilution of vaccine is plated in series onto 3 glycerol dextrose agar plates examine after incubation for 72 hours by the oblique light method for evidence of bacterial dissociation A vaccine containing more than 15% of " rough phase " colonies is discarded as being unsuitable for use

16 Four guinea pigs are injected intramuscularly with 1 0 ml of each batch of vaccine prepared These provide a safety test and are also used as a check on the virulence and immunizing power Two of the guinea pigs are killed at 11 days and the average number of brucellae per gram of spleen is estimated by serial culture in roll tubes The other two guinea pigs are left for nine weeks injected with a dose of 5 000 virulent *Br. abortus* (strain 544), and killed for spleen culture six weeks later These small numbers of guinea pigs do not of course, provide an adequate test of each batch of vaccine, but, since each batch is similarly tested, the combined results over the month or year do provide a sufficient measure

1 Dried culture is emulsified in a few drops of sterile saline and plated in series on to three glycerol dextrose agar plates, with the object of producing well isolated colonies Incubate at 37°C for 72 hours

2 Examine by the oblique light method[9] and select typical smooth type colonies Pick off colonies and transfer to potato infusion agar slopes Incubate for 72 hours

3 Examine cultures and emulsify growth in 5 ml of sterile saline Transfer this emulsion into 30 ml of sterile saline, which is sufficient to seed four Roux flasks of potato infusion agar Seed Roux flasks, allowing inoculum to flow over agar surface, and incubate at 37°C for 72 hours in the inverted position

4 Examine Roux flasks macroscopically for contamination, pour off residue of seed material and introduce 25 ml of sterile saline into each flask, agitate the flask until all of the growth is in suspension

5 Pour off suspensions into separate 100 ml Erlenmeyer flasks, test for purity, and examine microscopically Duration of purity test is seven days This suspension becomes the seed material for vaccine production

6 A convenient number of Roux flasks, each containing about 150 ml of potato infusion agar, are incubated at 37°C for four days as a purity check The agar is about 1 cm thick and has a surface area of about 200 cm^2

7 The seed material is diluted in a quantity of sterile saline sufficient to seed up to 200 Roux flasks Seeding is carried out under a hooded pipette Seed is allowed to flow over the agar surface, and the flasks are inverted and incubated at 37°C for 72 hours

8 Flasks are examined macroscopically The residue of the seed material is poured off and 25 ml of sterile buffered saline, pH 6 3, from an aspirating bottle fitted with a hooded pipette, is introduced into the flask, which is agitated until all growth is suspended in the buffered saline The suspension from three Roux flasks is then poured into a 300-ml Erlenmeyer flask, and a purity test is carried out on bulk suspensions and incubated for four days at 37°C

9 Examine cultures of purity tests and discard all contaminated suspensions The remainder are now bulked together in a 2 000-ml aspirating bottle This should be done by filtration and aspiration using a filter of glass wool contained in a 4 inch (10 cm) separating funnel The glass wool will remove any particles of agar in suspension

10 Remove 10 ml of pooled suspension and carry out purity test Examine film stained by Gram's method The remainder of the sample can be set aside for standardizing the vaccine The purity test cultures are incubated at 37°C for four days

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[9] See page 96

DISCUSSION: PART II

In response to many questions on laboratory procedure, attention was drawn to the reports on the first and second sessions of the Joint FAO/WHO Expert Committee on Brucellosis,* where these methods are discussed in detail. Requests for standard strains of *Brucella* for laboratory testing purposes, or for the International Standard Anti-*Brucella abortus* Serum, should be addressed to the Ministry of Agriculture and Fisheries Veterinary Laboratory, Weybridge, Surrey, England. Queries concerning strains, and dye and antigen requirements, may also be addressed to FAO or WHO, which will assist where possible.

In answer to a question as to whether a latent infection with *Brucella melitensis* in humans might play some part in the epidemiology of the disease, it was pointed out that only very rarely was the disease transmitted between humans; thus the problem was epidemiologically of little interest.

The question of possible climatic influence on the results of the agglutination tests in cattle was brought forward, but the discussion leaders had never encountered this phenomenon.

It was stressed that one of the main difficulties in the control of *Br. melitensis* in humans was the vast reservoir of infection among small ruminants, particularly goats. The eradication of *Br. melitensis* infection in livestock was much more difficult than that of *Br. abortus*. Prophylactic measures were easier to apply among cattle than among the small ruminants. In order to tackle the goat and sheep problem, there was a great need for an effective vaccine for these animals. Several FAO/WHO brucellosis centres were working on this problem.

The treatment of brucellosis in man was discussed, as being of great importance from both the medical and the economic viewpoints. The final aim of all treatment should be to protect patients from a relapse, as the intracellular habitat of the *Brucella* organism precluded, in most instances, its complete destruction in the body. The general opinion was that *antibiotic-sulfonamide combinations alone could not* give sufficient protection in this respect, except, perhaps, in infections caused by *Br. abortus*, the least pathogenic of the three types of *Brucella*. Combined vaccine and antibiotic therapy was discussed; it was felt that treatment always had to be decided upon on an individual basis, and that no fixed schedule for

* *World Hlth Org. techn. Rep. Ser.* 1951, 37; 1953, 67

of both virulence and immunizing power. Guinea pigs inoculated with strain 19 and killed at 11 days show an average spleen count of about 10,000 brucellae, compared with about a million for a virulent strain injected in a similar dose. The guinea pig protection rates for the years 1943–51 have been 72%, 75%, 77%, 70%, 79%, 68%, 67%, 72% and 70% respectively, indicating no decrease in protective power for guinea pigs, and it is known that these protection rates indicate a high and adequate immunizing power for bovines.

Preparation of Potato-Infusion Agar

Sound, raw, ripe potatoes are washed and pared, 250 g are sliced thinly into 1,000 ml of tap water, and this mixture is held overnight in a water bath at 60°C. Filter through muslin, allow the white precipitate to settle, decant off the supernatant fluid, and make up to 1,000 ml with tap water. To this add

Sodium chloride	5 g
Peptone	10 g
Beef extract	5 g
Glycerol	20 ml

Adjust pH to 7.6, add 25 g of agar powder, and autoclave at 15 p.s.i. (1 kg per cm²) for 20 minutes. Filter through a muslin pack containing absorbent cotton separated and turned in order to cross the cotton fibres. If the first filtrate is cloudy the process may be repeated. Add 10 g of dextrose to the filtered medium and mix well. Tube or bottle as desired, finally sterilizing at 15 p.s.i. for 20 minutes.

The culture media used for the purity tests are standard throughout: potato infusion agar slopes and dextrose broth containing Andrade's indicator in a Smith fermentation tube.

Fluids tend to encourage bacterial dissociation of this strain of *Br. abortus*. It is, therefore, desirable to use culture media which have been incubated for a few days, and also to incubate flasks in the inverted position.

Part III

LEPTOSPIROSIS

therapy could be recommended. Vaccinotherapy for *Br. melitensis* infection deserved consideration, particularly where the cost of antibiotics was prohibitive.

The problems of hypersensitivity to vaccine, and of whether eventual desensitization of patients who showed skin hypersensitivity was advisable from a therapeutic viewpoint, were then discussed. It was agreed that in some cases desensitization was advisable, and that in such cases it might be initiated with intradermic injections of diluted melitin (1%), repeated every other day with increasing doses. The same result could be obtained with a killed vaccine.

EPIDEMIOLOGY OF LEPTOSPIROSIS IN ITALIAN RICE-FIELDS

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Animals are the usual victims and hosts of predilection of *Leptospira*, man is attacked only incidentally, and only exceptionally may he act as a link in the chain spreading the disease

It follows that, for a proper understanding and explanation of the epidemiology of leptospirosis in man, it is essential to know how frequently and how commonly the leptospirae are present in animals, and to study thoroughly the circumstances which are likely to render these infected animals dangerous to man

Human beings are hardly ever infected by direct contact with the animal carrier, although cases of infection caused by rat bites, contact with the blood or entrails of crushed rats, and contamination from the urine of the rat, pig, or dog carrier, from the saliva of an infected dog, or from handling the flesh of an animal dead of leptospirosis, are not unknown in the literature

Infection usually takes place by indirect contact, the animal carrier eliminating with its urine leptospirae which can thus contaminate water, mud, or sometimes food In this way the organism may attack man It is well known that it does not survive drying, and that it is highly sensitive to pH variations away from the neutral, thus neutral, or almost neutral, reacting water and mud, in particular, are immediate sources of the infection in man

It is therefore of special interest to know how long pathogenic leptospirae can survive in water Here, however, our knowledge is incomplete, and is almost entirely limited to *L icterohaemorrhagiae* According to Chang and his collaborators,[4] this leptospira survives no more than 8-9 days in rivers, and a still shorter period if the temperature of the surrounding atmosphere is high Although not yet proved, it is, however, possible that other species of *Leptospira*, less sensitive than *L icterohaemorrhagiae*, can not only survive longer in water but also reproduce there This assumption is the only possible explanation for the highly infectious nature of the water of certain rice fields (see Babudieri [1, 2]) and of the mud covering certain pieces of ground after floods (Kathe [5])

leptospirae and the means of propagating the infection which it produces Other animals besides the host of predilection may become infected and even, occasionally, carriers and eliminators of the spirochaete, however, this is only an irregular and infrequent occurrence and so not very important from the epidemiological point of view Man, too, is only accidentally the leptospira's host, and with very few exceptions the infection begins and ends in the individual

To deal only with European species of pathogenic *Leptospira*, leaving aside a few types still much too little known, such as Poi, Sari, and Ghent, the leptospira's hosts of predilection are as follows

Type of Leptospira	*Host of predilection*
L icterohaemorrhagiae	*Rattus rattus*
	Rattus decumanus
	Dog
L canicola	Dog
L bovis	Ox
L pomona	Pig
L hyos (syn *mitis J*)	Pig
L bataviae	*Micromys minutus sorcinus*
L grippo typhosa	*Microtus arvalis*
L pyrogenes	Mouse
L australis A	Mouse (?)
L australis B	Rat (?)
L sejroe	*Mus musculus spicilegus*
	Apodemus sylvaticus
L saxkoebing	*Apodemus flavicollis*
L ballum	*Mus musculus spicilegus*
	Apodemus sylvaticus

It is easy to see why the leptospiroses propagated by field mice are contracted in the country and only in the places where the various species of Muridae live, why *L canicola* attacks only persons in contact with dogs, why *L icterohaemorrhagiae* is widespread everywhere (rats being found everywhere) and why *L pomona* and *L hyos* attack almost exclusively persons engaged in pig rearing A particularly eloquent case is that of infections by *L bataviae* This leptospira s main host is the *Micromys minutus sorcinus*, a tiny mouse to be found only in rice fields Cases of infection by *L bataviae* are therefore very common in rice fields, while no case has ever been recorded away from this special environment

One of the most obvious influences on the prevalence of the leptospiroses in general, and in particular on that of those transmitted by the Muridae, is the season In hot months, when work in the fields is at its height and there is also the largest number of persons bathing and fishing in fresh water, leptospirosis is naturally much more common than in the winter months Infection by *L bataviae* shows characteristics peculiar to that

The composition of the soil, and the flora and microflora in the water, also have important and sometimes quite complex favourable or unfavourable effects on the vitality of leptospirae—a fact which may account for the prevalence of human leptospirosis in certain regions and its total or almost total absence in others In view of the importance of water in the epidemiology of leptospirosis it is easy to see why these infections are particularly frequent in northern Europe and in the great river valleys where rain often falls and water abounds, while they are rather infrequent in the south where the climate is subtropical, with lower rainfall, and where water courses and accumulations of water are few and of limited size

The environmental conditions are particularly favourable for leptospirae in rice fields where expanses of semi stagnant tepid water are infested by a large number of small rodents, almost all of which carry and eliminate the spirochaetes The vast stretches of mud left by the great rivers of central and eastern Europe after floods also offer a suitable environment for the development of epidemics of leptospirosis, which are sometimes very widespread

The persons most exposed to infection are just those who are most frequently in contact with foul water, first of all, workers in rice fields and sewers, and then peasants in general, freshwater fishermen, road sweepers, miners and so on Cases of infection are also frequent among persons bathing in fresh water, among whom there have occasionally been veritable epidemics Stagnant or semi stagnant water infested by rats is particularly dangerous, especially near the outlets of sewers or drains

Cases have also been found among persons working in rat infested premises (millers, butchers, and workers in the fish preserving and cheese industries) Persons engaged in pig rearing are also very often exposed to infection

Many domestic and wild animals are likely to be naturally, or can be experimentally, infected with leptospirae, however, from the purely veterinary point of view—that is, in so far as they may provide clinical evidence of disease in useful animals—the only leptospiroses of interest are those of dogs, silver foxes, pigs, oxen, and horses There is no need to go into these infections here

On the other hand, we are interested, for purposes of human epidemiology, only in those infections which commonly make the animal a carrier and eliminator of leptospirae through the urine, and so a potential source of contagion to man The leptospiroses of interest from this point of view are those of rats mice, dogs, and pigs

Each species of *Leptospira* has its animal host of predilection, which is not only the animal most often infected but also the eliminator of the

leptospirae and the means of propagating the infection which it produces Other animals besides the host of predilection may become infected and even, occasionally, carriers and eliminators of the spirochaete, however, this is only an irregular and infrequent occurrence and so not very important from the epidemiological point of view Man, too, is only accidentally the leptospira's host, and with very few exceptions the infection begins and ends in the individual

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L australis B	Rat (?)
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L saxkoebing	*Apodemus flavicollis*
L ballum	*Mus musculus spicilegus*
	Apodemus sylvaticus

It is easy to see why the leptospiroses propagated by field mice are contracted in the country, and only in the places where the various species of Muridae live, why *L canicola* attacks only persons in contact with dogs, why *L icterohaemorrhagiae* is widespread everywhere (rats being found everywhere), and why *L pomona* and *L hyos* attack almost exclusively persons engaged in pig rearing A particularly eloquent case is that of infections by *L bataviae* This leptospira's main host is the *Micromys minutus soricinus*, a tiny mouse to be found only in rice fields Cases of infection by *L bataviae* are therefore very common in rice fields, while no case has ever been recorded away from this special environment

One of the most obvious influences on the prevalence of the leptospiroses in general, and in particular on that of those transmitted by the Muridae, is the season In hot months, when work in the fields is at its height and there is also the largest number of persons bathing and fishing in fresh water, leptospirosis is naturally much more common than in the winter months Infection by *L bataviae* shows characteristics peculiar to that

The composition of the soil, and the flora and microflora in the water, also have important and sometimes quite complex favourable or unfavourable effects on the vitality of leptospirae—a fact which may account for the prevalence of human leptospirosis in certain regions and its total or almost total absence in others In view of the importance of water in the epidemiology of leptospirosis, it is easy to see why these infections are particularly frequent in northern Europe and in the great river valleys where rain often falls and water abounds, while they are rather infrequent in the south where the climate is subtropical, with lower rainfall, and where water-courses and accumulations of water are few and of limited size

The environmental conditions are particularly favourable for leptospirae in rice fields where expanses of semi stagnant tepid water are infested by a large number of small rodents, almost all of which carry and eliminate the spirochaetes The vast stretches of mud left by the great rivers of central and eastern Europe after floods also offer a suitable environment for the development of epidemics of leptospirosis, which are sometimes very widespread

The persons most exposed to infection are just those who are most frequently in contact with foul water, first of all, workers in rice fields and sewers, and then peasants in general, freshwater fishermen, road sweepers, miners, and so on Cases of infection are also frequent among persons bathing in fresh water, among whom there have occasionally been veritable epidemics Stagnant or semi stagnant water infested by rats is particularly dangerous, especially near the outlets of sewers or drains

Cases have also been found among persons working in rat-infested premises (millers, butchers, and workers in the fish-preserving and cheese industries) Persons engaged in pig rearing are also very often exposed to infection

Many domestic and wild animals are likely to be naturally, or can be experimentally, infected with leptospirae, however, from the purely veterinary point of view—that is, in so far as they may provide clinical evidence of disease in useful animals—the only leptospiroses of interest are those of dogs, silver foxes, pigs, oxen, and horses There is no need to go into these infections here

On the other hand, we are interested, for purposes of human epidemiology, only in those infections which commonly make the animal a carrier and eliminator of leptospirae through the urine, and so a potential source of contagion to man The leptospiroses of interest from this point of view are those of rats, mice, dogs, and pigs

Each species of *Leptospira* has its animal host of predilection, which is not only the animal most often infected but also the eliminator of the

of stagnant or near stagnant lukewarm water rich in organic substances and rat infested and the necessity for workers to stay for long hours with their bare legs submerged in the water and mud are all conditions which are extremely conducive to the easy and frequent spread of infection

The same thing applies in the Italian rice fields which are for the most part situated in the plain of the Po especially in the provinces of Vercelli Novara and Pavia These rice fields cover a surface of about 150 000 ha and employ most of the local population nearly 200 000 workers a good many of the women come for the cleaning and harvesting of the rice from regions where rice is not cultivated In all it is estimated that in Italy no less than 600 000 persons are engaged in work in the rice fields The sowing of the rice takes place in April either straight into the rice field or in the special nurseries where the young plants are raised to be transplanted into the rice field in June After the sowing the ground is irrigated through a complicated system of canals having their origin in the rivers which cross the plain Towards the end of spring or at the beginning of summer most often between the end of May and the middle of July the important operation called the monda (cleaning) takes place The long rows of women with bare legs submerged in the water and river mud advance slowly separating the parasitic plants from the rice plants This is hard work often carried out in unhygienic conditions on some farms the cleaning is repeated twice in the season

It is particularly during the cleaning work that leptospirosis infections take place most easily A second dangerous period is during the gathering of the rice in September and October At this period the rice fields are dried up but at bottom they remain muddy and covered with pools of water Furthermore the ripe rice attracts small field rodents which sometimes become very numerous and constitute dangerous sources of infection

The study of leptospirosis in the Italian rice fields was started in 1937 by Dr P Mino [6] Dr L Bianchi and the author Their researches have been followed up in subsequent years and have led to a precise knowledge of the spread and characteristics of what I have called leptospirosis of the rice fields

In the Italian rice fields several serological types of pathogenic leptospirae exist and up to now the following strains have been precisely identified *L icterohaemorrhagiae L bataviae L pomona L sejroe L saxkoebing L australis B L grippo typhosa L hyos* and the types Poi and Sari

The frequency of these types (see fig 1) varies considerably The following tabulation gives the percentages of the different leptospiroses taken either from serological examinations made on 254 patients (see

organism in that there are two annual peaks one in May and June, the other in the September-October period These peaks coincide respectively with the rice-husking and harvesting periods

In central Europe the number of cases of infection by *L grippo-typhosa* also reaches a maximum in the autumn, because that is the season in which the main rivers are most frequently in flood

As regards the way in which the *Leptospira* enters the human body, penetration by way of alimentation or inhalation is quite exceptional, the usual route is through the skin and mucous membranes The *Leptospira* can enter through a healthy skin, particularly if it is softened by a lengthy sojourn in water, but the organism penetrates more easily through the small skin lesions common among persons who walk barefoot or work with their legs in water Entry is still easier through mucous membranes, which explains the frequency of leptospirosis among swimmers using the "crawl"—a style of swimming which requires the partial immersion of the head and thus involves the access of water to the mouth It is also interesting to note how frequent cases of leptospirosis are among persons who fall accidentally into rivers where the water is contaminated, and take in a certain quantity of water through the mouth or nose This observation has been made particularly in Paris for the Seine, and in Rome for the Tiber Here I would refer to a personal experience during the war when a lorry transporting German prisoners overturned into a ditch full of water Almost every prisoner who had taken in some quantity of water developed leptospirosis

Environment and means of infection can bring to bear an influence, as yet not clearly understood, on the progress and gravity of the disease For example, infection by *L icterohaemorrhagiae* contracted in the rice fields is almost always very slight, without icterus, and perfectly comparable with infection caused by the less virulent *L pomona* I have also observed—a fact which has been confirmed by Savino & Rennella,[8] working in Argentina—that whereas the majority of cases of infection by *L pomona* contracted by more or less direct contact with pigs show obvious meningeal symptoms, infections contracted in rice fields (Italy) or after bathing (Argentina) hardly ever show such symptoms

Thus, facts are observed which must be accepted, even though it is very difficult to supply any fully authenticated explanation for them They show that between the pathogenic agent and its environment there is quite a complex interplay of influences, the outcome of which can sometimes be observed, although its internal mechanism is not understood

It is known that the rice-field is the most favourable place for the spread of leptospirosis, particularly in the Far East The presence of vast stretches

In the second place we find *L icterohaemorrhagiae* propagated predominantly by rats and sometimes also by field voles or field mice A characteristic quite peculiar to this type of infection in the rice fields is that it is nearly always slight in character causing icterus only in exceptional cases We know on the contrary that in the cases of infection contracted outside the rice field icterus shows itself quite frequently

It is not known which animal is the carrier of *L australis B* and *L grippo typhosa* but it is probable that it is a field rat of some sort The type *australis B* has been met in Europe only in the rice fields it is known also that in the rice fields of the Far East *L pyrogenes* is often found and resembles very closely if it is not absolutely identical with *L australis B* *L grippo typhosa* very frequent in the rural parts of the countries situated to the north and east of the Alps is on the contrary very rare in Italy Probably this is due to the fact that *Microtus arvalis* the usual host of this species is not frequent in the rice fields

From among the cases caused by leptospirae of the group *sejroe saxkoebing* it is not easy to establish by serological examination which is the most predominant type in the rice field Some strains isolated by Mino in the Po valley have been found to belong to *L saxkoebing* in a rice field on the outskirts of Rome however I have isolated the type *sejroe* The two types in question are spread for the most part by species of *Apodemus* It is quite strange that in Denmark the infections caused by these types are nearly always mild cases whereas in Italy grave and sometimes fatal cases are found

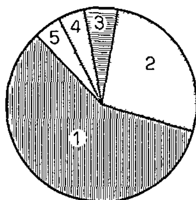
L pomona is less frequent in the rice fields but nevertheless in 1938 Babudieri & Bianchi [3] discovered 60 cases of infection caused by this type in a single rice field near Pavia

As the canals which irrigate the rice fields can sometimes be contaminated by discharge from stables it cannot be excluded that *L pomonae* eliminated in the urine by some host animals may infect the water of certain fields The fact however that in one case I succeeded in isolating *L pomona* from the kidneys of a rat leads me to believe that it is not impossible that even these small rodents propagate this type directly into the zones of rice cultivation

From two cases of illness in the rice fields Mino [7] isolated two strains one called Poi and the other Sari which up to now have not been exactly classified The type Poi is in every way related to *L javanica* These two types do not seem to be of great etiological importance in the clinical picture of leptospirosis in the rice fields

As far as the epidemiology of the infection is concerned it is evident that the leptospirae enter the water from the urine of the host animals They are able to survive for a shorter or longer period in the water and

FIG 1 FREQUENCY OF TYPE OF LEPTOSPIROSIS IN ITALIAN RICE FIELDS



1 = *Leptospira bataviae*
2 *Leptospira icterohaemorrhagiae*
3 = *Leptospira australis B*
4 = *Leptospira saxkoebing*
5 = Other types

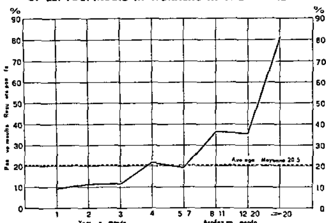
Mino [7]) or from examinations made on 509 apparently healthy "mondine" (workers engaged for the cleaning of the rice fields) (Babudieri)

Type of Leptospira	Percentage according to Mino	Percentage according to Babudieri
L bataviae	69 3	58 8
L icterohaemorrhagiae	19 3	22
L australis B	4 3	6 7
L sejroe saxkoebing	3 9	2 9
L grippo typhosa	1 2	5 8
L pomona	1 6	3 8
L Poi	0 4	—

The leptospirosis which is most frequently found in the rice fields is that caused by *L bataviae* About two thirds of the cases of sickness are due to this type of spirochaete In the Italian rice fields, *L bataviae* is spread particularly by *Micromys minutus sorcinus*, a very small mouse which makes its nest on the rice stalks This mouse is characteristic of the rice fields, where it is found sometimes in large numbers It is infected in a certain percentage of cases (about 25%, according to Mino [7]), and eliminates leptospirae in the urine, thus contaminating the water in the rice fields Species of *Apodemus* can also be a carrier for *L bataviae*

In Italy, *L bataviae* nearly always causes mild cases of the disease, in which icterus is very seldom found This form of disease is in contrast to the forms which, as we know, predominate in Java where *L bataviae* is strongly icterogenic

FIG 2 RELATION BETWEEN LENGTH OF SERVICE AND FREQUENCY OF LEPTOSPIROSIS IN WORKERS IN THE MONDA'



cases, one or two years after infection, the seroreaction for leptospirosis can give negative results In consequence, one is led to believe that, of those who work in the rice fields for a certain number of years, very few escape infection, even if this is not very often recognized or is inexactly diagnosed

In the prophylaxis of leptospirosis of the rice fields, disinfection of the water has been attempted, either by copper salts or by calcium cyanamide However, the results have not been very satisfying because of the high cost of the procedure and the very short duration of its effects, particularly in places where the water is running, however slowly

The fight against rodents in the rice fields, where they are very frequent and easily find nourishment, is very difficult in practice Attempts have been made to protect the workers by the use of rubber leg coverings and gloves This measure has also failed, either because the leg coverings are uncomfortable to wear for such long working periods and in the heat of summer, or because the gloves deprive the hands of the necessary sensitivity for the delicate cleaning work The use of protective ointments has also given equally negative results

It would, perhaps, be worth while experimenting with vaccination of the rice workers against leptospirosis as has already been done, with success, among miners in Japan I would again like to recall that, for the aims of social assistance, cases of leptospirosis among rice workers in Italy are considered in the same way as accidents at work, and it is more and more often advised that this illness be included in the list of occupational diseases

in certain circumstances, it is also possible for them to multiply It seems to me that only thus can the great frequency of infections observed in certain rice-fields where rats are not particularly in evidence be explained

The lukewarm water of the rice field, slightly pH alkaline, is favourable to leptospirae These are not uniformly frequent in the zones where rice is cultivated, but they are more or less plentiful according to the number of host animals, the condition of stagnancy of the water, and other causes which are not thoroughly understood

Meteorological factors also appear to influence the frequency of lepto spirosis, in the rice-fields, the hot, dry summers favour the frequency of infections

The leptospirae penetrate into the organism through the small cutaneous lesions to which the "mondine" are liable—it can be well understood that the work in the water macerates the skin which, when in contact with rough grasses, undergrowth, and stones, is easily abraded Certain types of eczema, susceptible to the penetration of leptospirae, can also be observed among the workers

From the clinical point of view, leptospirosis of the rice-fields, although it can be provoked by various species of *Leptospira*, presents a rather uniform picture In general, the infection appears in the form of "febrile leptospirosis" *with a very limited affection of the liver, kidney, and* meninges Headache and muscular pains often occur, congestion of the *subconjunctival vessels is very frequent, exanthema is observed* only in some cases, icterus is very rare There are quite frequently intestinal disorders in the form of colic types of dysentery, and ocular affections such as iritis are also often observed

Altogether, the illness takes a benign form and recovery is the rule However, leptospirosis in the rice fields has a notable economic importance, owing to the fact that, both at the critical stage of the illness and during convalescence, the considerable debility that it produces results in the loss of a great number of working days More, the possibility has not yet been probed that even mild cases of leptospirosis may cause, even after a long time, the morbid manifestations of a chronic type of disease

Leptospirosis of the rice-fields is an illness very widely spread in Italy Of 509 "mondine", apparently healthy and selected at random, I have found that 20 5% gave a positive *serological reaction for leptospirosis* If the "mondine" are subdivided into groups according to the number of years that they have worked in the rice-field, it is seen that in the oldest

The serological results give indications which are probably lower than the actual positive rates, for I have been able to establish that in several

LABORATORY DIAGNOSIS OF LEPTOSPIROSIS *

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In recent years laboratory methods for the detection of leptospiral diseases in man and animals have been established in many laboratories of clinical research As in other bacterial diseases, the laboratory diagnosis of leptospirosis is based on cultural and serological tests and animal inoculations Owing however to the special physical properties and growth requirements of leptospirae special techniques of investigation were developed during the early period of research

This short review is based on experiences in the leptospira laboratory in Amsterdam and in various laboratories in the tropics Most of the techniques discussed were developed by Schuffner and associates in the Netherlands and Indonesia Excellent descriptions of many aspects of laboratory investigations will also be found in general reviews on leptospiral diseases

Perhaps in no other bacterial disease is close co operation between the clinician and the laboratory worker so important as in a case of suspected leptospirosis Much depends on the appropriate material taken at the proper time by the clinician and on the choice of the best techniques of investigation by the laboratory worker In Weil s disease and other leptospiroses leptospirae may be present in the patient s blood during the first eight days of the disease and in rare instances some days later During that period these organisms also circulate in the cerebrospinal fluid However in human infections the number of leptospirae in the blood is generally very limited and positive findings by direct darkfield examination of a drop of blood are scarce although in severe cases of fatal Weil s disease leptospirae have been observed by this method In young guinea pigs and hamsters inoculated with the infected material, the number of leptospirae in the blood during the leptospiraemia may be much greater

During the first week of the disease investigation of a blood sample of the patient is always indicated the same can be said if a spinal puncture

* Abstracted from a monograph (in press) " on diagnostic laboratory methods in leptospiroses

Outside Italy, rice-fields are not very common in Europe I have no information from France on this form of leptospirosis, from Spain, I have learnt (Villalonga, personal communication) that, at least in Cata lonia, leptospirosis exists Three sera which have been sent to me from this region have given positive results for *L icterohaemorrhagiae*

I have given here a short survey of this illness, which is of particular interest in Italy, and concerning which considerable knowledge already exists, there are points, however, which must be clarified by pursuing researches in this field It is still necessary to study the possibility that leptospirae survive for a long time and multiply in the water of rice fields, to control the possibility of manifestations apart from this infection, to study the possible role of domestic animals in the spread of the disease, and, lastly, to keep in mind the eventuality that there still exist in the rice-fields unknown species of *Leptospira* Indeed, from time to time cases are observed which present clinically the complete picture of leptospirosis but which are, however, negative even on repeated serological tests

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As already mentioned, at this early stage direct darkfield examination of a drop of blood will occasionally give a clue for diagnosis by the detection of living leptospirae Unfortunately this technique is not infallible and also has the disadvantage that the inexperienced worker will easily mistake "pseudo spirochaetes" [5 6 17 21] (protoplasmatic filaments present in all suspensions of living blood cells) for true leptospirae, so that a false positive diagnosis can occur However, we would hesitate to abandon this simple method of investigation entirely, as at this stage of the disease the finding of actively moving leptospirae in the blood allows of a presumptive diagnosis of leptospirosis within an hour after receipt of the sample Negative findings have no diagnostic value as, in many instances, culture tests and animal inoculations carried out with the same blood sample in proved cases of Weil s disease have yielded positive results The reliability of this technique is enhanced by the modified centrifugal technique of Sieburgh Ruys [9 16] described in Annex 1 (see page 135)

Cerebrospinal fluid is treated in the same way as a blood sample without addition of an anticoagulant As expected positive titres in agglutination lysis tests will be much lower than blood serum samples, serial dilutions of $^1/_2$ $^1/_4$, etc of cerebrospinal fluid are recommended

Leptospiruria may occur in the second week of the disease and later, but the discharge of leptospirae in the urine is often very irregular and, if indicated, successive daily examinations of urine are needed As leptospirae are quickly immobilized and killed in the normally acid urine, little chance of a positive result is to be expected if the time between the voiding and the treatment of the urine in the laboratory is longer than at most a few hours To ensure optimum conditions, the urine should be alkalinized by giving the patient an alkaline diet one day before obtaining a sample of catheterized urine under strictly sterile conditions The sediment of the urine is subjected to darkfield examination Attempts to cultivate urine directly are generally unsuccessful The best method is to inoculate young guinea pigs intraperitoneally at the bedside with 1 2 ml of freshly voided morning urine The animals are then sent to the laboratory for further investigation and if the results are positive, heart blood is dispensed into tubes with culture media

Morphology, and Examination Technique

Leptospirae are slender organisms possessing very minute spiral coils Their length varies from 6 μ to 12 μ but much longer specimens may occur in old cultures They have a thickness of less than 0 25 μ and electron microscopic examinations even seem to point to a thickness of 0 1 μ

has been performed and a sample of cerebrospinal fluid is available In dispatching the sample to the laboratory, the day of illness on which the sample is taken should be stated Direct culture of blood samples taken during the first days of illness gives a high percentage of positive results but, if possible, inoculation of the material into guinea pigs or hamsters should not be omitted, as this will increase the chances of success At this stage, serological tests with the patient's serum will probably yield negative findings, although in some cases of Weil's disease weak positive titres have been recorded from the fourth day onwards Even negative serological findings at this stage may, however, be of indirect diagnostic value if at a later stage of the disease a repeated serological test is positive, it can then be concluded that specific antibodies have developed At this stage of the disease, examination of the urine for leptospirae is of little use

During the later stages serological tests are primarily indicated, while blood cultures and animal inoculations of blood will be of little value Leptospiruria may occur after the eighth day but special precautions are necessary for the investigation of urine samples In the cerebrospinal fluid and in the urine, leptospiral antibodies develop also in the course of the disease, but in much smaller quantity than in the blood

In a fatal case, after autopsy, blood, urine, and liver and kidney tissue should be investigated Sections of liver and kidney tissue, stained by Levaditi s technique, may show leptospira like structures, but no differential diagnosis of the special type of leptospira can ever be made in stained sections

Laboratory Procedures

The material received from a suspected case of leptospirosis can be treated by direct darkfield examination, culture tests, animal inoculation, and serological methods Each of these techniques has its merits and fallacies, and it will be the task of the laboratory worker to choose a combination of techniques which will give the best chances of success

It is recommended that the blood sample, taken during the first week of disease, be divided into two tubes, one without and the other with an anticoagulant (sodium oxalate or "liquoid Roche" is preferable to sodium citrate) After clotting, the serum in the first tube is used for serological tests, the second blood sample is used for direct examination, culture tests, and animal inoculation If only one blood sample without anticoagulant is received, the clot is triturated with a few drops of sterile saline, and the necessary tests are performed with this blood saline mixture, while the decanted serum is used for an agglutination lysis test

the advantage that cultures in these media can be stored for fairly long periods, but these semi-solid media are unsuitable for use in the agglutination lysis tests In recent years semi synthetic media have been developed for the study of metabolism and nutritional requirements These studies are still in their infancy but they have shown already that leptospirae consume oxygen, and their growth-rate is stimulated by small amounts of rabbit serum, haemoglobin, yeast extract, hydrolysed casein, and various amino-acids

When preparing media, the utmost care must be taken to ensure sterility of all glass-ware used and to prevent bacterial contamination of cultures or a change in the reaction of the medium Chemically pure reagents, non-corrosive glass ware, and fresh double-glass distilled water are recommended for use, while repeated control of the reaction of the different constituents of the medium is necessary

Pathogenic leptospirae are very susceptible to acid reactions The best growth is observed in buffered media with a pH of 7 2-7 4

Before using rabbit serum for culture media, it is advisable to test samples for the absence of leptospiral antibodies, as occasionally rabbits may become spontaneously infected with pathogenic leptospirae through contact with stray wild rats in the animal house If batches of these sera are added to the medium poor growth of leptospirae may occur

Our experience has been that not every brand of peptone is suitable for promoting the growth of leptospirae In the past, Witte's peptone has been universally used with good results, but this brand is at present not always available Of the newer, more refined, peptones, "proteose peptone No 3"[a] and "neopeptone" seem to serve instead of Witte's peptone In Stuart's medium[19] no peptone is incorporated

In all leptospira media ordinary bacteria grow copiously Thus, due care must be taken to prevent bacterial contamination of cultures, as in these instances the growth of leptospirae will nearly always be checked Purification of contaminated cultures by filtration through Seitz filters sometimes succeeds, but this is a cumbersome procedure, occasionally, success will be obtained by applying Schuffner's device of using a guinea pig as a living filter[12] 0 5 ml of the contaminated culture is inoculated intraperitoneally, ten minutes later heart-puncture is performed and the blood is inoculated into tubes with fresh culture medium

For all inoculations and subcultures a fair amount of material must be transferred—about 0 5 ml of a well-grown culture, and at least 3-5 drops of blood Good results are obtained by the direct inoculation of blood from a case of leptospirosis during the first few days of the disease Direct

[a] A Bacto-peptone obtainable from Difco Laboratories Detroit Mich USA

Leptospirae are practically invisible in bright light microscopy because of their extreme thinness Better visibility is obtained when they are examined by phase contrast microscopy, but by far the best way for the examination of fluid material is darkfield illumination For ordinary routine work, enlargements of 200×—400× are sufficient, but when structural details are to be examined, oil immersion objectives must be used When many consecutive examinations of fluid drops are to be made (control of cultures or readings of agglutination lysis tests), the low powered dry objectives have the advantage that the free distance between the objective and slide is great enough to permit of the examination of drops of fluid without a coverslip—a technique which speeds up the work considerably However, in a medium in which other material is present (cells, etc), a higher magnification of up to 400× is preferred, and the drop of fluid must be covered with a coverslip

Staining

Leptospirae are Gram negative organisms, but stain poorly with the usual bacterial stains Good results are obtained with Giemsa stain (see Annex 1, page 135), while in recent years other dyes (Victoria blue, mercurochrome) have been advocated With Burri's technique (a thin film of the material mixed with indian ink, nigrosine, or erythrosine) the contours of the leptospirae are also visible

In paraffin sections of tissues, leptospirae stain beautifully with Levaditi's impregnation technique, of which a variation, especially adapted to leptospirae, has been published [2]

It must be emphasized that, as far as present knowledge goes, no "species" diagnosis can be made on morphological and cultural characteristics of leptospirae alone All saprophytic as well as pathogenic leptospirae show the same morphology and cultural behaviour Although occasionally the detection of leptospirae in body fluids and tissues may have diagnostic value, further cultural and serological investigations are always necessary

Culture Procedures

The ordinary bacteriological culture media are not suitable for the growth of leptospirae, but well growing cultures can be obtained in buffered peptone and salt solutions enriched with inactivated rabbit serum and some other nutrients A number of fluid media have been developed which, after the organism has been cultured, can be used as antigens in serological tests Noguchi's semi-solid medium and its variations possess

animal before examinations are carried out Of other animals tried out, hamsters have proved to be susceptible to infections with *L canicola*, these organisms are only slightly pathogenic for guinea pigs White mice have also been used, but their reactions to various strains of pathogenic leptospirae are variable Moreover, spontaneous infection with *L ballum* (and perhaps with other types) may occur Unfortunately, an animal which is universally susceptible for all known strains of pathogenic leptospirae has not yet been found

In guinea pigs and hamsters the material (blood, urine, organ suspension) is inoculated intraperitoneally and from the third day onwards daily darkfield examinations of a drop of peritoneal fluid, obtained by puncture of the abdomen with a fine drawn out pipette, should be carried out When actively moving leptospirae are present, it may be assumed that leptospiraemia occurs Heart puncture is performed and the blood is dispensed into a number of tubes with culture medium If consecutive daily examinations remain negative during the first week after inoculation of suspected material, further examinations of peritoneal fluid are unnecessary, but then, in the second week and later, the blood serum is tested for the presence of leptospiral antibodies It is also recommended that daily temperature and weight control readings be taken If jaundice has developed, accompanied by a loss of weight and a drop of temperature, the death of the animal nearly always follows soon afterwards When these symptoms are present, it might be preferable to kill the animal and to perform an autopsy instead of waiting for its natural death When the interval between death and autopsy is too long bacterial contamination of tissues may occur Liver and kidney tissues as well as urine should be examined for the presence of leptospirae, and animal passage and culture tests should be performed in positive cases If inoculated animals remain alive, urine controls as well as agglutination lysis tests with the serum are recommended The observation of the animal must continue for at least *four weeks before a definite result can be reported*

Serological Tests

In cases of leptospirosis antibodies generally appear in the second week of the disease and increase rapidly, maximum titres are reached during the third or fourth week Thereafter, the titre diminishes gradually, but in Weil s disease and other leptospiroses residual titres may remain for many years afterwards

The agglutination lysis test is one of the most valuable diagnostic procedures Although in principle the same as in other bacterial diseases, the technique and interpretation of the reaction differ in some respects

inoculation in culture media of small amounts of tissue (liver or kidney) in which darkfield examination has shown the presence of living leptospirae is also often successful With a sterile scalpel a freshly cut surface is exposed which is then lightly scorched with a red hot spatula Particles of tissue material (in kidney tissue preferably parts of the cortex) are punched out with a not too fine Pasteur pipette and expelled into several tubes containing culture medium

Although the optimum growth temperature for leptospirae is 37°C better viability is observed at a temperature range of 30° 32°C In our laboratory all inoculations and transfers are placed for four days in an incubator at 32°C and thereafter stored at room temperature in the dark Occasionally the first growth may be detected (microscopically by examining a drop of culture fluid under darkfield illumination) after three days but usually later In fluid media growth is practically invisible macroscopically in semi solid media a white disc indicating a zone of maximum development appears about 1 cm below the surface level of the medium

Examination of primary cultures may be commenced on the third day and continued at weekly intervals If after one month no growth is observed the chance of the culture s becoming positive at a later date is negligible The majority of primary blood cultures from positive cases become positive in the second week after inoculation

Transfers of well grown cultures of leptospirae in fluid media should generally be made every three weeks a longer interval may be taken with cultures in semi solid media but regular control of all culture tubes is strongly advised as unexpected variations in growth density may occur

When cultures of different type strains of *Leptospira* are stored a foolproof system of registration and labelling is essential It must be remembered that serologically different types of leptospirae cannot be distinguished when in culture so that due care must be taken not to interchange cultures inadvertently during transfers The virulence of cultures of pathogenic strains is rapidly lost after repeated transfers and trials to restore virulence by animal inoculations seldom succeed

Animal Tests

Young guinea pigs (weight 150 200 g) are susceptible to inoculation with virulent strains of *L icterohaemorrhagiae* and a few other types of *Leptospira* The classical picture of a fatal infection with fever jaundice and haemorrhages in other leptospiroses however is often not fully developed so that the animal is only slightly ill or shows no symptoms at all This fact makes it inadvisable to wait for the death of an inoculated

and *L akiyami* must be included, in Australia, *L australis A* and *L hyos* (syn *mitis Johnson*), in south-east Asia strains of the Rachmat and pyrogenes groups, etc

The diagnostic evaluation of positive serological findings may be difficult when high co-reactions are found with other non causal strains in agglutination-lysis tests This may occur, for example, in cases of Weil s disease caused by the incomplete biotype, where high co reactions can develop for *L canicola* in the beginning of the antibody formation Usually these non-specific co-reactions decrease in titre during the course of the disease, and a second serum sample, taken some eight days later, will yield a high titre for the causal strain However, if only a single serum sample is available, this difficulty can be solved by agglutinin-absorption tests,[10, 13] the technique of which is described in Annex 1

Weakly positive agglutination reactions in single serum samples may, if there is no possibility of repeating the test at a later date, cause another diagnostic puzzle These reactions may be due to the early stage of the disease or to the presence of residual antibodies from a previous leptospiral infection In a few cases of Weil's disease, the appearance of positive seroreactions has been delayed, there are indications that this may be due to early treatment with immune serum or antibiotics In these instances, repeated serological tests at later periods of the disease may give a clue for diagnosis

Annex 1

LABORATORY TECHNIQUES

Staining of Leptospirae (Schuffner's Technique)

1 Make a thin film with the material to be invest gated (culture suspensions, etc)

2 Air-dry films without fixation

3 Flood the dried film with a strongly active solution of Giemsa stain, and stain for half an hour in an incubator at 37°C

4 Carefully wash off with tap water the stain and precipitate formed

5 Stain a second time with a fresh solut on of the same strength, for half an hour at 37°C

6 Rinse with water, and dry the slide in an upright position in a dust free place

Leptospirae are stained violet in well stained preparations the spiral coils are distinctly visible

When the test is carried out with a suspension of living leptospirae, both agglutination and lysis will be observed in positive reactions Generally agglutination is seen to occur first *in the lower dilutions of the serum* and is superseded by lysis at a higher titre At the end point of the reaction freely moving leptospirae are seen , in the next higher dilution the number of these is equivalent to the density in the control suspension with saline The technique of this reaction as developed by the Dutch school of Schuffner and associates [11 14 20 23] is described in Annex 1

Instead of suspensions of living leptospirae antigens of leptospirae killed by formaldehyde (final concentration 0 5%) have been used extensively [11 15] Lysis is then absent and the agglutination phenomenon will be observed to a titre usually slightly lower than when suspensions with living leptospirae are used Although this technique obviously has some advantages extensive comparisons of the two techniques have shown the superiority of the use of living antigens for the differential diagnosis of *human cases of leptospirosis and fo the analysis of the antigenic structure* of isolated strains With formolized antigens more non specific reactions tend to occur [27] However formolized suspensions might be of value in screening tests and are indicated in all absorption tests [10 13]

Some workers have devised a macroscopically visible agglutination reaction [4 7 18] using formolized antigens and small test tubes sometimes with a conical bottom in which agglutinated conglomerates of leptospirae clump together and become visible This method is not as sensitive as microscopic readings

Complement fixation tests have also been developed for leptospiral diagnosis [3] We have no personal experience with this technique but a study of the literature indicates that the results of complement fixation tests seem to be less specific while positive reactions occur somewhat later and at lower titres than in agglutination lysis tests *Recently the* reported results with a complement fixation reaction in which more refined preparations of antigens have been used seem to be more promising [1 8]

The result of a serological test may be the only positive laboratory finding on which the diagnosis of leptospirosis can be based It is therefore very important to improve as much as possible the reliability of the reaction by testing each serum sample with a number of antigenically different strains of leptospirae No fixed scheme can be recommended choice and number of type strains may vary and will depend on the scope of the investigation and on the known distribution of different kinds of leptospiral infections in the area from which the sample is taken

Reactions with type strains of leptospirae of which infections are known to be worldwide (*L icterohaemorrhagiae, L canicola L pomona*) will always be indicated in Japan reactions with antigens of *L hebdomadis*

6 Inactivate the tubes in a water bath at 56°C for from one to one-and a half hours

7 Check sterility by placing the tubes for 24 hours in the incubator at 37°C

Noguchi's medium (Dinger s modification)

Add to 100 ml of tap water 6 ml of a 3% agar solution Sterilize this solution and add 10% of inactivated rabbit serum

Serological Techniques

Agglutination lysis test

This reaction in the Amsterdam laboratory is based on a droplet technique using a special kind of Pasteur pipette and small porcelain plates instead of test tubes The same technique however can easily be adapted to small tubes and instead of one drop 0 25 ml may be chosen as the unit of volume

Each serum antigen mixture has a volume of six drops Serum dilutions are made with a solution of 0 85% sterile saline and mixed with equal volumes of a rich grown culture of a *Leptospira* type strain in Vervoort s modified medium Each serum sample is tested against a number of different type strains of *Leptospira* and for each strain and serum sample a separate plate and a sterile pipette are used Dilutions are made as follows the numbers indicate the number of drops expelled

Serum dilutions	diluting fluid	8	9	9	9	→ etc
	serum	2	↘ 1	↘ 1	↘ 1	
Test proper	diluting fluid	0	0	0	0	
	culture	3	3	3	3	
	serum dilution	3 ←	3 ←	3 ←	3 ←	
	titre	1/10	1/100	1/1 000	1/10 000	*Control*
	diluting fluid	2	2	2	2	3
	culture	3	3	3	3	3
	serum dilution	1 ←	1 ←	1 ←	1 ←	0
	titre	1/30	1/300	1/3 000	1/30 000	

Drops of diluting fluid are first run into successive depressions of the plate (or successive tubes) and then drops of the culture are added In between the pipette is carefully rinsed with diluting fluid and flamed Finally the serum dilutions are added and beginning with the highest dilution and working backwards the serum antigen mixtures are well mixed Sets of agglutination tests are incubated for 4 hours at 32°C The reaction is examined by taking up a drop of each dilution in a small short sterile loop after having whirled up the contents of each solution with the loop This drop is placed on a clean slide and examined with a low power dry objective (20× ocular 10×) and darkfield illumination

Agglutinin absorption test

1 100 ml of a formolized well grown culture of leptospirae in a fluid medium (final concentration of formaldehyde 0 5%) is centrifuged for 20 minutes at 10 000 r p m When the available high speed centrifuge has tubes of smaller volume, the 100 ml of formolized culture is divided into approximate fractions of 5 or 10 ml which are centrifuged at this speed

2 The supernatant fluid in all tubes is carefully pipetted off as far as possible and the sediment of all tubes is reassembled into one tube which again is centrifuged for

Duplo-Centrifugation Technique for Examination of Blood

1 To 10 ml of the blood sample 1 ml of a buffered[b] 1% solution of sodium oxalate is added This oxalated blood is first used for culture tests and 3 5 drops are added to each tube of culture medium

2 The remaining volume of oxalated blood is centrifuged for 15 minutes at 1 500 revolutions per minute (r p m)

3 A drop of the supernatant plasma is examined under a coverslip with darkfield illumination (enlargement approximately 400×) and the sediment is used for the inoculation of guinea pigs or hamsters

4 If darkfield examination is negative the plasma is again centrifuged for 20 minutes at 10 000 r p m

5 The supernatant plasma is carefully pipetted off and a drop of the sediment at the bottom of the tube is again examined under darkfield conditions

Culture Media

Modified Vervoort s medium

1 One litre of a 1/1 000 solution of a suitable brand of peptone (originally Witte s peptone) is made and boiled

2 Add 200 ml of Ringer s solution[c] and boil again

3 Add 100 ml of Sörensen s solution[d] pH 7 2 boil

4 Add 2 ml of a normal solution of phosphoric acid

5 Boil the mixture for 5 minutes a precipitate will be formed

6 Cool the mixture and filter through paper

7 The clear solution is sterilized for half an hour at 100°C

8 Dispense the solution in small sterile bottles or tubes 3 5 ml in each

9 Sterilize the bottles or tubes for half an hour at 100°C

10 Add 0 3-0 5 ml of rabbit serum (devoid of leptospiral antibodies) with a trace of haemoglobin to each bottle or tube

11 Inactivate bottles or tubes for half an hour in a water bath at 56°C

12 *Check sterility by placing the bottles or tubes overnight in the incubator at 37°C*

Korthof's medium

1 Dissolve in 500 ml of double-distilled water Witte s peptone 400 mg NaCl 700 mg $NaHCO_3$ 10 mg KCl 20 mg $CaCl_2$ 20 mg, KH_2PO_4 120 mg Na_2HPO_4 440 mg

2 Boil the solution for 20 minutes at 100°C in a Koch s apparatus

3 Filter through paper and dispense in tubes 5 ml per tube

4 Sterilize the tubes for half an hour at 100°C and cool

5 Add to each tube 0 4 ml of rabbit serum

[illegible] solution of d sodium phosphate d hydrogen phosphate [illegible]

[illegible] $NaHCO_3$ 0 01 g in [illegible]

d Sorensen s solution a mixture of 28 ml of a solution of KH_2PO_4 (9 078 g per litre) and 72 ml of a solution of Na_2HPO_4 (12 28 g per litre)

SEROLOGICAL CLASSIFICATION OF TYPE STRAINS OF LEPTOSPIRA*

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During the last decades the expansion of research in leptospiral infections in man and animals has created a worldwide awareness of the epidemiological and ecological importance of these diseases Many new leptospiral entities with definite host parasite relationships have been detected of which the causal strain of *Leptospira* was found to possess a structure antigenically different from types already well established

As clinical symptoms in many leptospiroses caused by different types of *Leptospira* may be closely similar, cultural and serological laboratory procedures have become more and more important Moreover, for the differentiation and classification of pathogenic strains of *Leptospira* the morphological and cultural characteristics are so similar that we must depend entirely on serological techniques Of these, agglutination lysis tests and cross absorption tests are at present basic tools of research and several studies on the classification of various strains of *Leptospira* have been published [27, 28, 31, 34, 36, 43, 48, 49, 52, 60]

Unfortunately however, differences in techniques and notation often make comparison and evaluation of results attained in different laboratories exceedingly difficult

In 1950 at the Fifth International Congress for Microbiology, in Rio de Janeiro, I pointed out some of these divergencies and made a plea for international co operation in establishing standard methods of typing procedures and other laboratory techniques in *Leptospira* research [55] My proposal resulted in the nomination of a Leptospira Sub-Committee of the Nomenclature Committee of which some members now are engaged in surveying these different problems

Although at present our knowledge about the antigenic fractions in leptospirae is still rather incomplete I believe that agglutination lysis

* This work has been partly aided by a grant from the Central National Council for Applied Scientific Research in the Netherlands T N O

10 minutes at 10,000 r p m The supernatant fluid is now pipetted off and discarded and the final sediment containing the leptospirae is used for the test proper

3 Nine parts of this sediment are mixed with one part of the immune serum (or the patient's serum, depending on the purpose for which the absorption reaction is needed), diluted to a standard titre of 1/3,000 In cases where the original titre of the patient's serum is lower, the serum is used undiluted

4 The immune serum or patient's serum is left in contact with the leptospira suspension for at least 24 hours at room temperature and then centrifuged for 5 minutes at 10 000 r p m

5 The supernatant serum is carefully pipetted off and used in the necessary agglutination tests with formolized leptospira antigens As the serum has already been diluted 10 times, the first dilution in these tests will be 1/30 (see the technique recommended for the agglutination lysis test, page 137)

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Again, the same value will be obtained if, in another laboratory carrying out the same test but using an antiserum with a homologous titre of 1/10,000, a titre of 1/100 is observed I believe that this notation would greatly facilitate comparison of results

As an example, tables I and II show the old and new notations of the serological characteristics of strain Sentot, taken from table 7 of the study by Walch Sorgdrager et al [51]

TABLE I SEROLOGICAL CHARACTERISTICS OF STRAIN SENTOT OLD NOTATION *

Strain	Seroreactions		
	tit e with stra n Sentot and immune sera	homologous t tre of serum	t tre with immune serum Sentot and strains
Wijnberg	1/1 000	1/30 000	1/10 000
Kantorowicz	1/300	1/30 000	1/3 000
Dog Utrech t IV	0	1/30 000	0
Moscow V	1/30	1/30 000	1/3 000
Sejroe M 84	0	1/10 000	0
Swart van Tienen	0	1/10 000	0
Rachmat	1/10 000	1/30 000	1/10 000
Sal nem	0	1/100 000	0
Dog HC	0	1/30 000	0
Djasiman	1/1 000	1/10 000	1/100 000
Benjamin	0	1/30 000	0
Sentot	1/300 000	1/300 000	1/300 000
Sarm n	1/100	1/100 000	1/30
Bat 90-C	0	1/100 000	1/1 000
Naam	1/1 000	1/300 000	1/10 000
3705	0	1/30 000	0
Rat Batavia 46	0	1/10 000	1/30
Rat Semarang	1/30	1/10 000	1/30
Hebdomadis	0	1/30 000	0
Ak yami A	1/10 000	1/30 000	1/10 000
Andaman A	0	1/10 000	1/10
Bal co	0	1/30 000	0
Pomona	1/300	1/30 000	1/3 000

* After table 7 of Walch Sorgdrager et al [4]

In table II the evaluation of the results of the different agglutination-lysis tests is much easier than in table I We now see at a glance that strain Sentot shows affinity to the *autumnalis* group and to strain Djasiman Although it will be observed in table I that the titres of strain Sentot with other immune sera (for instance, with the immune serum of the strain Naam) are rather high (1/1,000), this value is only 0 3% of the homologous titre of immune serum Naam with its own strain (1/300,000) and therefore must be considered a "co-reaction"

I want here to point out explicitly that the results of agglutination lysis tests alone are never sufficient to analyse completely the antigenic structure of a strain under consideration, for a final analysis, cross absorption

tests and cross absorption tests carried out by standardized techniques (at least until better methods have been developed) give sufficiently constant results to be used as a basis for *typing procedures* *It seems inadvisable* to use complement fixation tests or protection tests in animals for the typing of strains, as additional variables are then added In order, however for the results of the two basic tests to be easily reproducible and comparable, three conditions must be fulfilled

(*a*) the use of rigidly controlled " standard " type strains [a] and immune sera ,

(*b*) a standard technique and

(*c*) a practical notation of the results

Using these principles, I have worked out a provisional scheme of classification of type strains based on the assembled data derived from studies made in the Leptospira Research Laboratory at Amsterdam during the last 20 years These studies by Schuffner and associates (Walch *Sorgdrager, Ruys, Bohlander, Gispen, Wolff, and others*) have only been partly published After the last war, investigations were continued, and in our classification many unpublished data have been incorporated of which more details will be published later

Strains of type cultures were subcultured in a modified Vervoort s medium and were regularly controlled as to growth capacity and antigenic structure In our experience this structure of parasitic leptospirae has *been remarkably stable* Notwithstanding the fact that some strains have been held in culture for fifteen years and more, we have never seen any indication of a spontaneous major change

The agglutination lysis tests were carried out with suspensions of living *Leptospira* in absorption tests, however, formolized suspensions have been used throughout As to technique,[b] both tests have been performed according to the work of the Dutch school [29 35 37, 38, 43 53, 54]

The results have been recorded according to the following rules

(*a*) type strains and corresponding immune sera are arranged in a *fixed order* ,

(*b*) the agglutination titre is expressed as a percentage of the reciprocal of the titre *of the strain with its homologous immune serum*

Thus, if an antiserum with a homologous titre of 1/30,000 gives a titre of 1/300 with a strain under examination, this value is put down as 1

[a] The authentic ty of the standard strains must be absolutely safe There is evidence in ... Petersen Broom and van der Hoeden

tests are necessary But our notation yields a clear picture of strains showing affinity to the strain under test, and the respective immune sera with which absorption tests are indicated can be easily located

TABLE II SEROLOGICAL CHARACTERISTICS OF STRAIN SENTOT NEW NOTATION

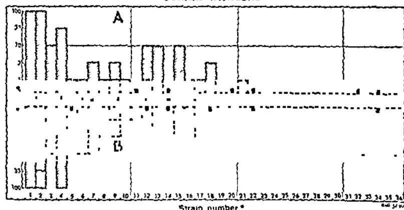
Serogroup of *Leptospira*	Strain	Seroreactions*	
		with strain Sentot and immune sera	with immune serum Sentot and strains
icterohaemorrhagiae AB	Wijnberg	3	3
icterohaemorrhagiae A	Kantorowicz	1	1
naami	Naam	0 3	3
canicola	Dog Utrecht IV	0	0
benjamini	Benjamin	0	0
schuffneri	Bat 90-C	0	0.3
grippo typhosa	Moscow V	0 1	1
hebdomadis	Hebdomadis	0	0
medanensis	Dog HC	0	0
wolffi	3705	0	0
sejroe	M 84	0	0
autumnalis AB	Akiyami A	33	3
autumnalis A	Rachmat	33	3
sentot	Sentot	100	100
djasmani	Djasman	10	33
pomona	Pomona	1	1
australis A	Ballico	0	0
andaman A	CH 11	0	0 003
bataviae	Swart van Tienen	0	0
pyrogenes	Salinem	0	0
javanica	Rat Batavia 46	0	0 01
semaranga	*Rat Semarang 173*	*0 3*	*0 01*
sarmini	Sarmin	0 1	0.01

* Expressed as a percentage of the reciprocal of the titre of the strain with its homologous immune serum

Finally, the notation allows the construction for each strain examined of a characteristic ' agglutination graph " by depicting the different percentage values as columns of different height along a *fixed sequence* of type strains Reactions of the strain with different immune sera are denoted on the upper side of the base line, projections downwards represent *the reactions of the immune serum of the same strain* with all the other strains, ranged numerically in the same order Some examples are given in fig 1, 2, and 3

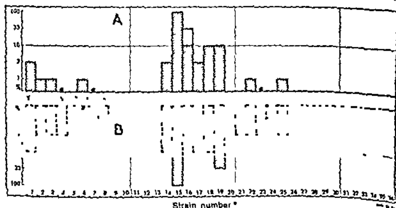
The results of the typing of thirty six strains of *Leptospira* in the new notation are assembled in table III The recorded titres are mean values of the results of two or more tests carried out on different occasions during the last fifteen years This series of strains contains representatives of the causal organisms of several well known leptospiral diseases Also included are a number of strains isolated in Indonesia by the writer in different periods before the second World War and by other workers in Sumatra and Java These strains were preliminarily typed locally, sent to the

FIG. 1. AGGLUTINATION GRAPH OF LEPTOSPIRA ICTEROHAEMORRHAGIAE AB, STRAIN WIJNBERG



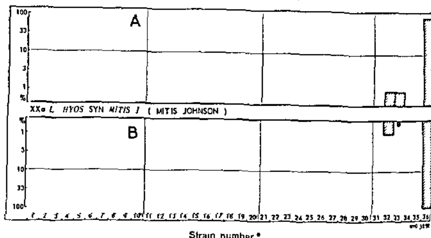
A — reaction of strain Wijnberg with immune sera of other strains
B — reaction of immune serum Wijnberg with other strains
— Percentage value of agglutination titre of strain with immune sera and of immune serum with other strains
— different values in repeated tests
* — titre of 0.1% or 0.3%
* The numbers of the strains correspond to those given in table III

FIG. 2. AGGLUTINATION GRAPH OF LEPTOSPIRA SENTOTI, STRAIN SENTOT



— different values in repeated tests
* — titre of 0.1% or 0.3%
* The numbers of the strains correspond to those given in table III

FIG 3 AGGLUTINATION GRAPH OF LEPTOSPIRA HYOS (SYNONYM MITIS J) STRAIN MITIS JOHNSON



A = reaction of strain Mitis Johnson with immune sera of other strains
B = reaction of immune serum Mitis Johnson with other strains
▨ = Percentage value of agglutination titre of strain with immune sera and of immune serum with other strains
• titre of 0 1% or 0 3%

* The numbers of the strains correspond to those given in table III

Amsterdam laboratory for a final analysis, and deposited in our collection of type cultures

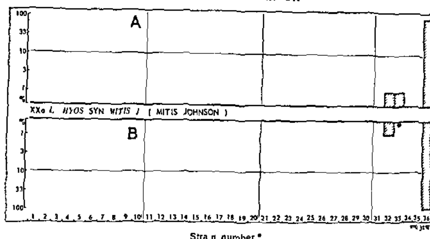
The clinical and epidemiological importance of infections caused by *the Indonesian strains is not restricted to Indonesia*, in latter years, human infections caused by leptospirae antigenically identical with, or closely related to, members of these groups have been found with increasing frequency in other parts of the world Some additional data on the isolation of these strains are assembled in table IV [c]

Collier [10] has classified these strains as separate entities Although *this may have been premature, we have provisionally adopted his* nomenclature

The strains in question are arranged in twenty groups A first indication for the arrangement was furnished by the agglutination-lysis pattern The second step was the performance of cross absorption tests with immune sera of all closely related strains For the final classification, the difficulty arose of determining the criteria for grouping If, after absorption, all antibodies in the immune serum of a strain are reciprocally absorbed by

[c] More detailed information on these strains will be found in a monograph by the author of this paper to be published in the near future

FIG 3 AGGLUTINATION GRAPH OF LEPTOSPIRA HYOS (SYNONYM MITIS J) STRAIN MITIS JOHNSON



A reaction of strain Mitis Johnson with immune sera of other strains
B = reaction of immune serum M t s Johnson w th other strains
= Percentage value of agglutination t tre of strain with immune sera and of immune serum with other strains
• = titre of 0 1% or 0 3%

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[c] More detailed information on these strains will be found in a monograph by the author of this paper to be published in the near future

another strain, we may conclude that the two strains are serologically identical, if on the other hand after absorption, no specific antibodies against the homologous strain are absorbed the two strains have no serological identity

But if only part of a t tibody is absorb d the decision whether the strain in question is to be classified in the c group as the strain from which the antiserum w prepared bec n c much more difficult. In accordance with previou rk carried ou the Amsterdam laboratory by Schuffner, Walch Se lrager and oth rs we have provisionally adopted the following cr r on two stra are considered to be heterologous if the antiserum of each strain after rption by the other strain retains at least 10% of it nal titre whe retested against the homologous strain [d]

Table III is arranged twofold tab Vertically, under the number of the strain, are recorded the titres agglutination lysis reaction of its immune serum w th suspensions of ll other strains, horizontally are recorded the titres of each strain wit the other immune sera.

As we are of the opi n that our present knowledge of leptospirae is insufficient to define the criteria on wh ch the subdivision of the genus should be based, we have not designate the different type strains as "species", a term which has a very defin te meaning in zoological and botanical nomenclature Our designation type strain" and "group" implies no further consequences The classification leaves room for ultimately fitting other strains into one of the groups if this should prove necessary This classification should by no means be accepted as final. It may well be that further research and consideration will necessitate changes in grouping or in sequence of groups [e] Strains of different groups may ultimately be combined or further subdivision may be necessary. It is hoped, however that this scheme of classification may form a basis for future constructive work

It will be observed that this series of type strains of Leptospira is not complete A number of aberrant strains, already mentioned in the literature, are not included Studies on some of these strains are in progress Unfortunately, several other strains, stated for ... to differ serologically from L icterohaemorrhagiae and other strains ...

TABLE IV. DATA ON STRAINS OF LEPTOSPIRA DISCUSSED

Strain	Serogroup of *Leptospira*	Origin	Remarks	Bibliographical reference no
1 Wijnberg	*icterohaemorrhagiae AB*	Amsterdam, 1926	Complete biotype, from urine of case of Weil's disease, 11th day of illness	4 16
2 Kantorowicz	*icterohaemorrhagiae A*	Amsterdam	Incomplete biotype, from blood of case of Weil's disease, 2nd day of illness	4 16
3 Naam	*icterohaemorrhagiae*	Medan Sumatra, 1938	From blood of human case of leptospirosis febrilis, 4th day of illness	51
4 Mankarso	*icterohaemorrhagiae*	Medan, Sumatra 1938	From blood of human case of leptospirosis febrilis, 2nd day of illness	
5 Rat Batavia 46	*javanica*	Djakarta	From *Mus brevicaudatus* (carrier)	15
6 Pol	*javanica* (provisionally)	Vercelli Italy, 1941	From human case of rice field leptospirosis	25
7 Sarmin	*sarmini*	Sumatra	From guinea pig inoculated with blood of human case, 4th day of illness	21
8 Bat 90-C	*schuffneri*	Djakarta 1938	Via inoculated guinea pig from brain of *Cynopterus* sp	11 12
9 Dog Utrecht IV	*canicola*	Utrecht, 1931	From urine of dog with fatal leptospirosis	19 33
10 Benjamin	*benjamini*	Medan Sumatra, 1937	From blood of human case of leptospirosis febrilis 2nd day of hospitalization	51
11 S 102	*ballum*	Amsterdam, 1941	Type strain (Mus 127) isolated by Borg Petersen in 1943 from urine of carrier *Mus musculus spicilegus* Strain S 102 was isolated from urine of white mouse	7, 59
12 Salinem	*pyrogenes*	Sumatra, 1924	From guinea pig inoculated with blood of case of leptospirosis febrilis	26 45
13 Zanoni	*australis B*	Australia, 1937	From case of benign leptospirosis Schüffner's assumption that Zanoni might be the incomplete biotype of *L pyrogenes* could not be confirmed although both strains possess a common antigenic fraction	24 58
14 Bat 3868	*cynopteri*	Djakarta, 1938	From kidney of bats Strains 3522 C and 3868-C are serologically identical	12
15 Sentot	*sentoti*	Medan Sumatra, 1937	From blood of fatal case of leptospirosis febrilis on 2nd day of hospitalization	51
16 Akiyami A	*autumnalis AB*	Japan, 1925	Complete biotype, from blood of patient with seven-day fever	20 36, 48

TABLE IV DATA ON STRAINS OF *LEPTOSPIRA* DISCUSSED (*continued*)

Strain	Serogroup of *Leptospira*	Origin	Remarks	Bibliographical reference no
17 Rachmat	*autumnalis A*	Sumatra 1923	Incomplete biotype, isolated from blood of guinea pig inoculated with patient's blood on 2nd day of illness	1 36 53 54
18 Bangkinang I	*autumnalis*	Sumatra 1923	From liver of guinea pig inoculated with patient's blood on 2nd day of illness	39
19 Djasiman	*djasiman*	Sumatra 1937	From blood of guinea-pig inoculated with patient's blood on 15th day of illness	21
20 Ballico	*australis A*	Australia 1937	From case of cane fever	24
21 *Munchen C 90*	*australis A*	*Germany* 1942	*Strain received from* Professor Rimpau was isolated *from a human case* of leptospirosis	56
22 Pomona	*pomonae*	Australia 1937	Causal organism of swine-*herd's disease* (Gsell)	9 30
23 Moscow V	*grippo typhosa*	Moscow 1928	From human case of mud fever in Russia	40 41 48
24 Duyster	*grippo-typhosa*	Amsterdam 1942	From human laboratory infection caused by the bite of *Microtus arvalis*	56 58
25 Bernkopf	*grippo-typhosa*	Israel 1947 (?)	From human case of leptospirosis	2 56 58
26 Hebdomadis	*hebdomadis*	Japan 1918	From human case of *nanukayami*	17 46
27 *Dog HC*	*hebdomadis (L medanensis)*	Medan Sumatra 1929	From a clinically normal dog (*carrier*)	22 23
28 3705	*hebdomadis (L wolffi)*	Medan Su*matra* 1927	From blood of human case of leptospirosis febrilis	36
29 Hardjoprajitno	*hebdomadis*	Sumatra 1938	From *blood* of human case of leptospirosis febrilis	56
30 M 84	*hebdomadis (L sejroe)*	Denmark 1937	From *blood* of *human* case of benign leptospirosis	4 8
31 Mus 24	*hebdomadis (L saxkoebing)*	Denmark 1942	*From kidney of Apodemus flavicollis* (carrier)	5 8
32 van Tienen	*bataviae*	Djakarta 1932	The original strain Swart *isolated* by Walch has been lost Strain van Tienen is *serologically* identical	13 14 46, 47
33 Paidjan	*bataviae*	Sumatra 1938 (?)	From human case of lep*tospirosis febrilis* Serologically slightly different *from strain van Tienen*	56
34 *Rat Semarang 173*	*semaranga*	Java 1937	From *Rattus rattus brevicaudatus* (carrier)	30
35 CH 11	*andaman A*	Andaman Islands 1931	From human case of leptospirosis	42
36 Mitis Johnson	*hyos (synonym mitis J)*	Australia 1940	From blood of human case of *leptospirosis* The strain is serologically identical with *L hyos*[a]	18

have been lost Among those are strains Ghent,[8] Kebler and Tuyen Quang,[44] and Mukingilwa [27] Therefore renewed studies on the classification of these strains have become impossible, and the original observations on the antigenic structure now have only historical value Schuffner s ' *Spirochaeta icterohaemoglobinurica* ' [32] was never obtained in pure culture

To minimize the chances of losing type strains of *Leptospira* it is strongly recommended that serologically aberrant strains should be deposited in one of the collections of type cultures of *Leptospira* from which controlled subcultures can be made available for workers in other parts of the world

The ever increasing number of antigenically different pathogenic leptospirae calls for a well planned combined study on an international scale in different *Leptospira* research centres A first step in this direction has now been made by the work of the Leptospira Sub Committee of the Nomenclature Committee set up at the Fifth International Congress for Microbiology

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DISCUSSION · PART III

The discussions opened with some statements about the occurrence of leptospirosis among the different domestic animals In Switzerland, serious outbreaks have been reported among horses and pigs The rate of infection in the different animals varies somewhat, but has been found in as much as 80% of all pigs within certain herds The disease is concentrated in areas where pigs are reared upon waste products from butter and cheese factories Pigs do not seem to suffer clinically from the disease, and they generally recover quickly They can, however act as transmitters of the disease for at least eight to nine months

From Tunisia it was reported that leptospirosis is rather severe among cattle and that they often succumb to the disease This is true, also, of the USA, where, in addition to fatal cases abortions very frequently occur.

In Austria, the frequency of canine leptospirosis was said to show some correlation with the number of dogs in the area This was rather noticeable during and just after the second World War when a remarkable decrease in the number of dogs was followed by a corresponding decrease in canine cases of leptospirosis After the immediate post war period, the number of dogs increased rather quickly and a sudden rise in canine cases of leptospirosis started in 1948 and continued until the end of 1951, there was a regression in 1952 In blood tests on 880 dogs, using the agglutination lysis test, 362 gave positive reactions Of these cases, 247 were positive to the *Leptospira canicola* group It was remarkable that only two human cases of leptospirosis were diagnosed among the owners of 7,370 leptospirosis infected dogs Examination of the personnel in the veterinary clinic handling these dogs revealed not a single case of leptospirosis

The question of elimination of leptospirae in the urine was discussed It was pointed out that this process could continue for a rather long period in different animals In dogs, it might not start until eight or nine days after the onset of the disease, in some cases it might not occur at all It was usually difficult to isolate the organism unless special precautions were taken as to the acidity of the urine Of 258 dogs with positive serological reactions, *Leptospira* strains were isolated from only 77 Leptospirae were sometimes obtained in pure culture

In connexion with laboratory procedures, several participants in the discussion stressed the desirability of having central laboratories from

which *pure Leptospira strains* and, *if possible*, positive control sera could be distributed Dr J W Wolff and Dr B Babudieri declared themselves prepared to undertake the distribution of *Leptospira* strains to the different diagnostic laboratories which required them

In a number of cases, it was found that dogs treated with antibiotics gave no positive serological reactions to leptospirosis, in spite of very suspicious leptospirosis symptoms

The use of vaccine against leptospirosis in man had not always given good results although a certain effectiveness had been reported by some workers, particularly in Japan Killed vaccine could be used, but only in highly exposed groups

The use of penicillin in canine leptospirosis seems to be of little value while other antibiotics such as streptomycin have proved to be effective In cattle the application of streptomycin and aureomycin has given *promising results To be effective, antibiotics should be given early in the* course of the disease, in both man and animals

Leptospirosis in horses is now believed to cause the disease known as ' periodic ophthalmia In central and eastern Europe it is also believed to be the cause of Z dar disease, which is characterized by gastro enteric and encephalitic symptoms The possibility was pointed out that some cases now diagnosed as infectious anaemia of horses in reality represent leptospirosis

Part IV

Q FEVER

EPIDEMIOLOGY, DIAGNOSIS, AND PROPHYLAXIS OF Q FEVER

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First identified in Australia in 1937 Q fever has since been found successively in the United States of America and in the countries of southern and central Europe during and after the second World War

In recent years, the disease has also been reported in Central America, in several regions of Africa, and in the Near East Research has shown that the disease exists, at least among animals, even in South America (Babudieri & Parodi[4]) and eastern Asia (Kitaoka—personal communication) This seems to indicate that the disease is, in fact, universal, although its existence in certain countries has not yet been confirmed in spite of the research conducted to that end The Scandinavian countries are of this group

Q fever does not bring about a definite symptomatology in the animal, nor does it cause any considerable damage to the zootechnical industry, with the possible exception of the rare cases of abortion in goats (Kilchsperger & Wiesmann[18]) To the veterinarian, Q fever is therefore of indirect interest only

The frequency with which the disease occurs in man differs from country to country and is still difficult to evaluate In Italy, for example, which has a population of some 47 million, it is estimated that in 1950 1 there were about 20,000 cases of the disease of which only a small number were diagnosed and recognized

Victor et al ,[29] working in the Los Angeles region where the disease is endemic, have found, by using their very sensitive but not yet fully verified opsonic reaction, that 31% of the healthy individuals submitted to the test gave positive seroreactions The disease is particularly common among certain workers such as shepherds, butchers, and veterinarians, even though it may not always be diagnosed accurately

In man, Q fever is most frequently localized in the lungs (atypical pneumonia), sometimes it is found elsewhere—or at least there are morbid symptoms involving other organs and systems (brain, kidney, liver,

testicles, circulatory system, etc) The death-rate among white people is very low, about 1% or even less, but it is higher among the indigenous peoples of equatorial Africa (Jadin & Giroud [17])

Epidemiology

As a rule, Q fever occurs in short epidemic outbreaks affecting small towns, villages, and sometimes even small, isolated groups of cottages in the country It affects some tens, and sometimes hundreds, of persons breaking out suddenly, and occurring a second time or more after a certain period has elapsed , it does not spread but rather remains confined to the epidemic area The disease sometimes takes on an endemic character with the appearance, from time to time, of isolated and quite unconnected cases In general, this is frequently the case where the pasture is rich and where a high percentage of animals contaminated by *Coxiella burnetii* comes to graze It is therefore in the country that the disease takes this form , in Sicily, for example, this *type of dissemination* predominates

However, in large towns, such as Rome and Florence, where rare and isolated cases are reported among persons who have never left the town and have never been in contact with animals which may have been carriers of the pathogenic agent, the disease takes on endemic characteristics Q fever rarely reaches epidemic proportions in large towns This has occurred only once, immediately after the war, in the 1945-8 period, in Palermo, Florence, and Munich These outbreaks took place at a time when Q fever was still almost unknown in Europe, and were very imperfectly studied , it is therefore understandable that *C burnetii* should have been held responsible for certain cases of different etiology Instances in which the contagion is due to the proximity of carrier animals or to rickettsia contaminated material, should be considered separately

In order to appreciate clearly the often clouded aspects of the epidemiology of Q fever, two elements in particular must be considered the powers of resistance of *C burnetii* and its diffusibility, and the sources of infection and means of dissemination of the pathogenic agent

C burnetii is one of the asporogenic organisms with the highest resistance to physical and chemical bactericides At ambient temperature it resists drying for over four weeks It also survives for several days in water and in milk It can withstand a temperature of 50°C for at least 30 minutes, and often temperatures of 60°C and 70°C for shorter periods Dried, and exposed in a thin layer to the rays of a germicidal lamp, it will survive for 30 minutes , it will withstand the action of formol at 0 5% for 24 hours and of ethanol at 50% for five minutes and over The

antibiotics which have a therapeutic action on Q fever do not succeed in sterilizing the organism, even in the case of cure Not only has *C burnetii* the above mentioned powers of resistance but it is also very small, and therefore capable of remaining suspended in the air for long periods and of being carried in the wind It will easily be realized that if a considerable quantity of rickettsiae contaminate the air, they are capable of causing several cases of infection even a very considerable time after their dissemination

It is very important to know the reservoirs of the virus Passing over other regions such as Africa and Australia, where it seems that wild animals are reservoirs and confining our attention to Europe, we note that *C burnetii* is to be found in a few domestic animals, in particular cattle and sheep It is difficult to say how frequently these animals are infected and infectious, in view of the differences existing between the various countries and regions and because the criteria adopted to identify cases of actual infection cannot be accurately compared So far, the methods used in this research have generally been serological, allergy tests seldom being used, and the positive results obtained have always been construed as showing a state of actual or at least, potential infectiousness

The experience of recent years has shown that this is not so, for not only have there been instances of animals giving seropositive results while having no coxiellae in their organism but there have also been instances of animals with negative serological reactions acting as reservoirs of this pathogenic agent However although it is no longer possible to decide whether or not an animal is infectious by relying solely on serodiagnosis, the value of these tests in the identification of the disease should not be underestimated Experience has also shown that where there are outbreaks of Q fever in man, there are also outbreaks among domestic animals, and that if the latter are subjected to serological or allergy tests positive results will certainly be obtained It is not true that the animals giving these results invariably excrete *Coxiella*, but the results will confirm the existence of an infection in the group of animals or in the building concerned Diagnostic, serological and allergy research on large numbers of animals and in various localities is still very valuable from the point of view of epidemiology, even bearing in mind the reservations set out above

[illegible]

tives among sheep and 33% among cattle

In our research on animals from several epidemic areas, we have found sheep 19 3% positive and cattle 22%. Even the equidae (horses asses)

probably how many of the cases of the disease in rural Australia and the USA come about In Europe, however, the part played by ticks is insignificant, especially in the infection of human beings The disease is not only more common there in winter, when ticks drop off their animal hosts, but is also fairly common in the Swiss and Italian alpine valleys, where ticks are practically unknown It is possible that in southern Europe, where ticks are numerous, these arthropods may be of some importance in the transmission of the disease from animal to animal, particularly in large flocks of sheep, but this has not yet been proved conclusively Contaminated ticks (*Hyalomma savignyi*) have been found in Spain and once in Italy

With regard to flies as possible vectors of Q fever, our research has shown that the fly can become infected and act as carrier for about one month Even the larva of the fly can be infected through its food, but the rickettsiae disappear at the time of the metamorphosis The part played by the fly is not yet clear , it seems likely that such a role exists, but is not of great importance in spreading Q fever So far no one has written on the question of whether other arthropods may be responsible for spreading the disease, although some have been successfully infected experimentally It must also be borne in mind that *Coxiella* is present in the blood for a rather short time The blood of the human being generally becomes a source of infection during the first week of illness The researches of Smith & Derrick,[27] Blanc et al ,[3, 10, 11, 12] Bell et al[8] and Lennette et al ,[19, 20] and our own work,[2, 3, 5] have revealed that the rickettsiae can be identified in the blood of experimentally infected animals before the seventh day, never later, and that sometimes they cannot even be found during the early days

One of the ways in which *Coxiella* may be eliminated is through the kidneys, although it is very rarely found in the urine we have found it only once in a ewe six days after it had been experimentally infected

The faeces may be rich in rickettsiae (Bell et al[8]) but here lack of research prevents the mention of any hypotheses as to how frequently *Coxiella* may be eliminated in this way As already admitted above, dogs can be infected through the digestive system, and for some days the rickettsiae can be isolated, living and virulent, from the excrement

I have already mentioned that *Coxiella* can be found in the sputum of patients Lennette et al[19, 20] have found it once only in the nasal secretion of a ewe infected the same day by intravenous injection The elimination of *Coxiella* in the milk is much more important A large number of research workers have shown—a fact subsequently confirmed by us—that both cattle and sheep, if infected, eliminate *Coxiella* in their milk over very long periods

Certain animals may become infected long before lactation, and may begin to eliminate the coxiellae months later when giving their first milk Moreover, Lennette et al have found *Coxiella* in the udder secretion of a ewe six weeks after infection in the absence of a true milk secretion

It has also been found that sometimes only a part of the udder becomes infected and that experimentally the animal can be locally infected without the infection spreading throughout the organism or to the parts of the udder close to the site of inoculation As a rule, *Coxiella* are regularly eliminated in the milk, but sometimes in irregular quantities Animals eliminating the rickettsiae in milk usually show a seropositive reaction which may sometimes be strong However there are many cases where rickettsiae are eliminated by completely seronegative animals, just as there are others where it is impossible to isolate them in milk given by animals which are certainly seropositive The lacto-seroreaction of animals eliminating *Coxiella* may be either positive or negative

In Europe there has been no research complete enough to give a satisfactory indication of how many animals eliminate rickettsiae in their milk In California, however the problem has been given thorough study and the results obtained are impressive According to Luoto, Huebner & Stoenner,[21] 10% of the cows in the Los Angeles region eliminate *Coxiella* in their milk and 40% 50% of the 60 000 animals coming into the region each year become infected within six months and eliminate *Coxiella* Indeed, 90% 95% of the cows having a complement fixation titre of more than 1/32, which shows a recent infection, give milk containing *Coxiella* In view of the great importance of this problem to health, it would be worth while undertaking similar research in Europe

Women may also eliminate *Coxiella* in milk I have myself on one occasion isolated *Coxiella* in the milk of a woman who had become infected seven months earlier and had given birth about a month before

From the epidemiological point of view one of the most important ways in which rickettsiae are eliminated is in the placenta The research of Kilchsperger & Wiesmann,[18] who found the placentas of goats very rich in rickettsiae, has been continued, and the virus has been found to be present in enormous quantities in the placentas of ewes and cows

Luoto et al [21] found that of 33 placentas from cows not less than 1 4 positive on serodiagnosis, 13 were contaminated, Welsh et al [30] found that 15 of 43 placentas (35%) from seropositive ewes were contaminated, while 6 of 29 placentas (21%) from seronegative ewes contained *Coxiella*

The placenta may contain enormous numbers of rickettsiae, one gram of placenta may often contain as many as one million times the amount required to infect a guinea pig (Welsh et al [30])

This indicates that these placentas can be a very dangerous source of infection, especially as they are abandoned everywhere, on the ground, in farm buildings, on pasture-land, and on dung hills, sometimes devoured by dogs, sheep, or cows, or else dried up and reduced to dust In fact many isolated cases of infection, and even epidemics, are originally caused by an infected placenta Not only the placenta, but other uterine secretions which accompany or follow delivery can give rise to infection, they are sprinkled on the ground, in farm buildings, or on roads where the animals have passed, to become a pestiferous dust in the hot season In this connexion I would add that I have been able to isolate *Coxiella* from the placenta of a woman who had become infected six months before, and had been treated with very large doses of aureomycin, chloramphenicol, and oxytetracycline [a]

Coxiella can even survive for a very long time in the organs of animals and particularly in the udder, spleen, liver, and kidneys, so that it is understandable that butchers can become infected by handling infected material I have seen a case of two workers being infected by carrying a contaminated liver from one place to another Cases of Q fever are, moreover, very common among laboratory workers Chicken embryos containing rickettsiae are highly dangerous, and the use of infected mem branes carries with it a definite danger of infection during the preparation of antigens

What happens to the *Coxiella* when it reaches the external environment after elimination through one or other of the channels mentioned above? According to the circumstances, the rickettsiae may either be eliminated in the placenta and devoured by any of several animals which become infected and in their turn eliminate the virus in their stools, or they may be eliminated in urine, stools, and all other forms of excretion, which in due course dry and become a dust taken everywhere by the wind

Rickettsiae in milk are absorbed with the milk or, alternatively, may contaminate butter, cheese, and cream They survive for several days and even several weeks in such foods However, we have found that the *Coxiella* in cheese is no longer a source of infection after 46 days

We now come [illegible]
We have already [illegible]
tance in spreadir [illegible]
are the most us [illegible]
The many cases of experimental infection of man studied by Blanc et al ,[9, 10, 11, 12] Fonseca et al ,[14] and Giunchi,[15] observation of what happens in nature, and results obtained by the experimental infection of animals,

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[a] *Oxytetracycline* is the non proprietary name for Terramycin

provide sufficient information on how infection takes place and on the relative importance of the above mentioned channels. It is now proved that if the virus has penetrated by inhalation there will be pulmonary localization of the disease, and that there will not be pulmonary localization if *Coxiella* has infected the organism through food, by subcutaneous injection, or through the surface mucous membranes.

From the point of view of epidemiology the respiratory tract is much more important than the alimentary tract as a means of access for the infection, because almost all cases of Q fever in Europe show pulmonary localization. Infection through the alimentary tract is always possible—we have experienced it ourselves—provided that large quantities of rickettsiae are absorbed, but that is very difficult. This being so, contaminated raw green foods may be relatively important while for purposes of epidemiology, milk and milk products are of major importance. However, it would seem that milk although often contaminated, is not the main source of infection in California. Huebner et al.[18] have admitted, after carefully studying 300 cases of infection that only 32% of patients drank unboiled milk. On the other hand the fact that in the County of Los Angeles, where this research was carried out, only 5% of the population habitually drink raw milk shows that milk may in reality be a source of infection although in a relatively small number of cases, and that the problem of freeing milk and milk products from *Coxiella* should be given serious consideration. Recently a number of isolated cases in Florence and Catania (Italy) in which there was no pulmonary localization, and where the patients had had no contact with animals or the countryside, indicate that infection through the alimentary tract may occasionally be of some practical significance.

However, it is generally agreed and most of the outbreaks which we ourselves have studied indicate that the disease is mainly carried through the air. In almost all these cases patients had been in contact with animals carrying or eliminating the virus or, in the case of epidemics, living near infected farm buildings or frequenting places where groups of animals fed or passed.

The outbreak in Chiaravalle (a small town near Ancona, Italy) is very instructive from this point of view: during the winter of 1949 about 100 persons developed Q fever. 80% of the patients lived on or had shops opening onto, the two roads used daily by a flock of contaminated sheep going to and returning from pasture. Of the remaining 20%, not living on these two roads, several frequented a cafe very often visited by the shepherds of the flock and their dogs. The outbreak during the winter of 1950-1 at Maccarese near Rome (46 verified cases) was similar: all the patients lived on a country road used every day by a group of contaminated animals going to pasture.

Observations, by American workers during the second World War,[28] of the epidemic outbreak at Pagliana (Italy), where 40% of the soldiers sleeping on straw contracted the disease while only 4 7% of the others were infected, and the well known cases in Switzerland and the USA among workers unpacking machinery padded with contaminated straw, show that the disease is most commonly contracted by inhalation

Another probable way in which the virus may penetrate is through the conjunctiva We have carried out experiments on animals and have demonstrated, although with some difficulty and irregularity, that they can become infected in this way provided that large quantities of *Coxiella* are used This means of access seems unlikely to be very important under ordinary circumstances

Many external factors may promote or prevent the spread of Q fever When this is realized it is not difficult to see that in dry windy weather *Coxiella* can spread through the air with ease, while rain will prevent its spread During the epidemic outbreak in San Marino, Babudieri & Suzzi Valli [7] showed how atmospheric conditions might influence the dissemination of *C burnetii*

The presence of flocks of sheep near dwellings and, worse still, their passage between them are other factors promoting the spread of the disease During the summer when, in Italy at least, the animals are taken to graze in the mountains in deserted or almost deserted regions, there is a considerable decrease in the number of cases, while in winter, when they come back to the plains and to the neighbourhood of dwellings, the number of cases increases

The isolated cases, observed from time to time in towns or in the country, which cannot be attributed to contact with an infected animal may perhaps be attributed to pathogenic organisms carried by pigeons, or other birds or animals

To sum up the above considerations—it seems that the research carried out in recent years has shed some light on the epidemiology of Q fever, revealing the close relation between the disease in man and its presence in animal carriers, sheep, cattle, and sometimes birds It has also shown the danger arising from the parturition and milk of infected animals, it has shown that, in Europe at least, the part played by blood sucking arthropods in spreading the disease is very small, while helping us to recognize the importance of inhalation as a means of access for the virus Yet in the face of all this new knowledge and progress we are obliged to admit that there is still something in the epidemiology of Q fever which has escaped us, something that we have not been able to grasp—the problem of why seropositive animals should remain harmless for a long time and then suddenly, for no apparent reason, become highly infectious for a fairly

long period ? Again, one cannot see why there should have been many epidemic outbreaks in Italy and Switzerland during the winters of 1949-50 and 1950-1, while in the winter of 1951 2 these outbreaks almost disappeared, without any change in the environmental conditions These phenomena, however, may perhaps be ranged among the largely unknown causes which govern and determine the outbreak and disappearance of all epidemics

Another interesting problem which has so far been little studied is the way in which Q fever is transmitted from animal to animal, especially where there are few or no ticks

Diagnosis

Q fever in man can be identified clinically but must be confirmed by laboratory analyses, in animals clinical examination is not enough to reveal possible suspects and only laboratory work can be decisive The research and diagnostic techniques necessary for the identification of Q fever are considered later (see page 193) here the value and importance of this research is discussed

The diagnosis can be taken as definitive if *Coxiella* can be isolated in t blood and sputum of the patient It must, however, be borne in mi that not every positive serological or allergic reaction indicates the sur and certain presence of the disease, it may only be that the individual u question has been in contact with *C burnetii* more or less recently Yet by using several of the known methods of diagnosis we can often give a sufficiently accurate answer to the practical questions which arise in deciding whether *C burnetii* is or is not the cause of the disease under consideration In order to be able to give this opinion, it is necessary to be thoroughly acquainted with the results to be expected from the various serological reactions, from the beginning of infection to the years following cure

The reaction most often used is the complement fixation reaction It is positive in a minority of cases (less than 9% according to Lennette et al [19, 20]) during the first week of illness during the second week it rises to 65% positive, to reach 100% during the third week Even where the reaction is already positive in the first week, the titre is hardly ever high enough to make the result conclusive, it is only after the second week that the complement fixation is of any real practical significance The positive reaction will appear later, often taking more than 30 days, if the patient has been subjected to early treatment with antibiotics effective on *Coxiella*, and there will be the same time-lag if the infecting quantity was very small, the latter observation is based on experience with animals infected with milk which sometimes contains very small numbers of coxiellae,

The complement fixation titre reaches its maximum one or two months after the beginning of the illness, and then slowly declines until it becomes negative The positive reaction may continue in man for a long while, we have found it in individuals who had contracted the disease five years before This phenomenon is not common, often the titre falls within two months

Antibiotic treatment does not affect the titre of a positive seroreaction

In animals the complement fixation reaction continues for a shorter period we have observed that a rather high titre became negative in experimentally infected sheep at between 200 and 220 days

As already shown above, a positive complement fixation reaction does not mean that the person in question is a carrier, or, worse still, a disseminator, of the disease The organism can be spread even by a person who has become seronegative, just as it can be no longer present in an individual still seropositive

This shows that the positive nature of the seroreaction is a consequence of the organism's defence against the penetration of the virus and not of a persistent infection, even in a latent form

As regards the interpretation of low-positive titres, it is necessary to adopt the criteria generally used for the evaluation of serodiagnostic reactions Each case must be considered separately in some cases a low titre may have a certain significance, while in others a high titre may be of no importance However, the increase of the titre between the first and second of two blood samples taken at an interval of a few days may be highly significant in diagnosis

Subject to all reservations, we would suggest that 1/4 should not be taken as positive, that 1/8 should be considered doubtful, and that only 1/16 should be looked upon as really positive

As regards the agglutination reaction, the various authors are not unanimous The general view is that this reaction takes place earlier but does not continue as long as the complement fixation reaction It seems that this rule is not constant and that there are many exceptions, agglutination gives different results according to the antigen and method used If the antigen and method which I favour are used, positive agglutination will be seen to take place within a few days of positive complement fixation but to continue longer As this phenomenon does not always go according to rule, it is advisable to use both reactions in the serological diagnosis of Q fever, just as the Wassermann test is ordinarily used together with a flocculation reaction for the diagnosis of syphilis

As regards allergy tests, it is not yet known at exactly what date from the day of infection the reaction becomes positive, in the guinea pig it is

the 25th day—a time when serological reactions are already positive Consequently this reaction cannot be used for early diagnosis but only in epidemiological research

An interesting property of the allergy reaction is that it will give clearly positive results where the serological reaction has already become negative Moreover, the eyelid reaction sometimes brings about an ephemeral reappearance of deviating antibodies in the blood at a fairly high titre, whereas in healthy animals the allergy reaction provokes only the appearance of agglutinin, and never that of deviating antibodies in the blood Our knowledge of the opsonic reaction is not yet sufficient to allow of a sound evaluation

It should be stressed that a positive serological or allergy reaction may only mean that there has been contact between the organism and *Coxiella*, and not necessarily that the organism is actually infected or infectious Furthermore, the passage from positive to negative reaction does not necessarily indicate loss of immunity the lower limit adopted in determining whether a reaction is to be taken as positive or not is always conventional, or at least conditioned by our technical possibilities, the organism may still be immune below this limit and may often be able to ward off a subsequent attack unless exposed to too powerful a source of infection

Prophylaxis

The prophylactic measures which can be taken against Q fever have two aims the protection of man against contamination from the animal carrier or from infected material and the direct suppression of the sources of infection

A first step which every country should take is to make cases of Q fever in man compulsorily notifiable Of course, the clinical diagnosis almost always requires serological confirmation, hence the necessity for each country to have a certain number of well equipped laboratories to carry out the necessary tests

Compulsory notification of cases in animals is not feasible in view of the absence of clinical symptoms and the difficulty of making analyses in order to find out whether or not an animal is a carrier or eliminator of rickettsiae

The possible existence of animal carriers must be borne in mind in connexion with the notification of cases of the disease in man, so that

and how frequently the disease occurs in domestic animals The agglutination reaction might be very suitable for this research

The following are the first precautions to be taken to prohibit the housing of animals in dwellings, to prohibit the movement of groups of animals through towns or past dwellings, and to make the disinfection of vehicles used for the transport of sheep and cattle compulsory The destruction of placentas should be compulsory, they should never be left on the ground, and dogs should never be allowed to devour them A study might also be made of the possibility of isolating the infected animal, or at least of limiting its movements for a period of about 20 days after delivery, that being, according to Rosati's research,[24, 25] the period for which it may still be infectious As we have no practical way of ascertaining whether the animal is still eliminating *Coxiella* or not, the application of all these precautions to given flocks or single animals is no easy matter If, however, a case of the disease in man is seen to be directly connected with the presence of an infected animal or group of animals, the necessary precautions might be taken, ranging from the disinfection of farm buildings, and the sequestration or isolation of the group suspected, even to compulsory slaughter

In Italy, it has been decided that groups of animals not permanently confined to farm buildings must have a "health book" in which all developments connected with health are to be recorded, so that the anamnestic indications likely to be of use in any diagnosis are always available These precautions naturally apply more to those groups of animals which are not permanently confined to farm buildings, and which are the most common and dangerous sources of Q fever infection

Other useful precautions are the control of flies and the de-lousing of animals

It is more difficult to take precautions against possible infection from birds, and in any case the importance of birds in epidemiology is not yet accurately known As regards foods, steps should be taken concerning milk and milk products As explained above milk is often infected and *Coxiella* has a particularly high resistance to heat Various research workers have reached different conclusions on the degree of this resistance, probably because they have adopted different methods From the practical point of view the research carried out in California is the most interesting Huebner et al and Lennette et al have carried on research separately, each using naturally infected milk and the installations commonly used for pasteurization

According to Huebner et al,[16] a temperature maintained at 71 5°C for 15 minutes is sufficient to destroy *Coxiella* In some cases, it seems, the rickettsiae will survive at a temperature of 61 5°C for 30 minutes According to Lennette et al,[19, 20] pasteurization at 61 5°C for 30 minutes destroyed

the rickettsiae in 34 out of 35 samples of milk In the latter, the phosphatase reaction was positive, which probably indicates defective pasteurization A temperature of 74°C for 17 minutes has proved insufficient to sterilize 2 out of 42 samples treated

Rosati,[24 25] in Italy, has shown that a temperature of 63°C for 30 minutes cannot destroy *Coxiella* in his opinion, a temperature of 80°C maintained for one minute is necessary

It would be well to repeat and continue this research Nevertheless, the results are already sufficient to convince us that low-temperature (61° 63°C) pasteurization is not enough and should be abandoned, as is the present tendency

Special precautions should be taken before authorizing the consumption of raw milk It would be wise to have the animals submitted first to the allergy test and then to a serological test taking advantage in positive cases of the speedy and strong immunity reaction Everything said of milk also applies to milk products, with the exception of dried cheeses which, even if contaminated, are naturally free from infection after 30-40 days

As regards prophylactic measures to be applied to man, I consider that they should not be over rigorous in view of the extreme rarity of contagion from man to man It is nevertheless advisable to isolate the patient and to disinfect the surroundings Major precautions should be taken in the case of women, where there is danger of the rickettsiae being eliminated in the placenta and in milk Care should be taken to see that the placenta does not infect those present at the delivery, and that placentas from human suspects are strictly controlled before use for Filatov therapy as practised in several countries

It would also be advisable to test the milk of women nursing infants not their own There is no danger in an immunized mother nursing her own child, as the latter will also be congenitally immune Blood collected from the umbilical cord and sometimes used for transfusions must be carefully tested this precaution is necessary because the cord can easily become infected by contact with the placenta, the blood itself will not carry the infection

Of the more drastic precautions, the slaughter of infected or suspect (seropositive) animals cannot be given serious consideration for very obvious economic reasons, except in special cases where the animal has become a really dangerous eliminator of *Coxiella*

The use of antibiotics for the sterilization of infected animals is also impracticable in view of the enormous expense involved, the results would very probably not be at all satisfactory Here I would mention a case which I observed myself of a woman whose organism could not be completely sterilized, even by very strong doses of aureomycin, oxytetracycline,

and chloramphenicol The attempts of Luoto et al [21, 22] to use intra mammary and intravenous injections of aureomycin to suppress the viruses in cows eliminating *Coxiella* in milk have nearly always failed

One possibility remains—that of prophylactic vaccination, which should be carried out while the animal is young and before it has con tracted the disease Even here there is an economic difficulty, vaccine and vaccination being costly Moreover, it is not known whether the vaccine would in fact protect the animals completely and prevent them from taking an infection which, though slight and asymptomatic, could make them disseminators of the pathogenic organism

To my knowledge, there has been only one experiment so far in the vaccination of domestic animals It is that of Luoto et al , in 1952, with 147 cows then placed in an infected environment together with a control group of 139 non-vaccinated cows Only 9 5% of the cows with completely seronegative reactions before vaccination contracted the disease, as against 51% of the control group 27% of the non vaccinated cows began to eliminate *Coxiella* in their milk, while none of the vaccinated animals did so

These experiments, although not conducted under the best conditions, are nevertheless satisfactory and worth repeating and continuing

There are no grounds for the fears expressed by certain authors that the seropositive reaction due to vaccination might possibly be wrongly interpreted as the result of an infection, thus impeding future epidemiological research Vaccination gives rise only in very few cases to the appearance of antibodies

The possibility of vaccinating human beings might be considered, particularly for certain categories of laboratory workers exposed to infection Vaccination has been practised both in the USA and in Europe with satis factory results, as far as immunity is concerned It must, however, be borne in mind that anti Q fever vaccine retains a certain degree of toxicity and, in certain individuals, has had detrimental side effects (e g , haemor rhagic nephritis) Every precaution must therefore be taken in using it

In conclusion, the possibility of effective Q fever prophylaxis depends mainly on accurate knowledge of the epidemiology and dissemination of the disease However, all these necessary precautions must be taken to prevent direct contact between man and an animal considered a possible source of infection, particularly in the dangerous period during and after delivery Special attention must be paid to milk and milk products Temperatures certain to be effective should be used in pasteurization At present we have no pharmaceutical products capable of sterilizing the contaminated organism completely Prophylactic vaccination of animals, although as yet imperfectly tested, is promising, even though its use is still conditioned by the very high production costs

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PREVALENCE OF Q FEVER IN EUROPE AND SURVEY METHODS FOR ITS DETECTION

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Several authors [3, 23, 30] have reviewed fairly recently the known information on the prevalence of Q fever throughout the world The prevalence of the disease in Europe, based on information from published reports and on a survey conducted under the auspices of the World Health Organization, is summarized in one section of the present paper

It is of interest to note that outside Europe Q fever has been reported in the following countries Australia Panama, United States of America, Algeria, Belgian Congo, Egypt, Libya Morocco, Union of South Africa, Ceylon, China, Cyprus, India Israel Japan, and Malaya Presumptive evidence of Q fever infection has also been found in Mexico, French Equatorial Africa, and possibly Iraq

In recognition of the need for more information on the epidemiology and prevalence of Q fever throughout the world, the World Health Organization has sponsored a survey in 28 countries The methods recommended for use in this survey are given in section 2 of this paper The results of the survey obtained in the European countries are incorporated in section 1, along with information from the published reports which have already appeared in various journals

1 PREVALENCE OF Q FEVER IN EUROPE · SURVEY RESULTS AND PUBLISHED REPORTS

The following countries of Europe are at present taking part in the Q fever survey and results have been received from those marked with

Q fever infection was first diagnosed in Turkey in 1948[26] throughout widely separated areas of the country The following results were obtained from a survey

Sera	*Number of tests*	*Number of positive reactions*
Human	868	256
Bovine	149	48
Buffalo	41	2
Sheep	49	31
Goat	41	11

Yugoslavia

Simovic et al[32] reported on the first outbreak of Q fever in Bosnia in August 1950, where 12 cases in human beings were confirmed The source of infection was not definitely established In the WHO survey, 130 sera, mostly from sheep, have so far been examined (September, 1952), of which 14 positive sheep sera have been confirmed Several positive cases in human beings have also been established

United Kingdom of Great Britain and Northern Ireland

Stoker[34] first reported on Q fever in Great Britain in 1949 when the [illegible]

appeared in various counties, the highest incidence being in Kent and the next in Devon[24] In 1951, Harvey et al[17] described an outbreak among 27 students at a college in Kent A few students showed signs of symptoms suggestive of enteric or *Salmonella* infection, and only one case of lung involvement was observed Slavin[33] has recently described the epidemiology of Q fever among cattle in Great Britain as revealed by a country-wide survey Cattle blood samples sent to the laboratory for routine diagnosis and blood samples from guinea pigs inoculated with milk for the detection of the tubercle bacillus were examined for antibodies It was shown that 2 1% of cattle have antibodies for *C burnetii*, and the guinea pig samples represented 6 9% infected farms in England, 2 0% in Wales, and 0 8% in Scotland

2 SURVEY METHODS FOR DETECTION OF Q FEVER

Several methods can be used to obtain evidence of endemic or enzootic Q fever The basic laboratory procedure we shall describe here is the complement fixation test, this is given in detail later in this paper The agglutination test is also useful but requires much more antigen Q fever

In the Republic of San Marino, Valli[36] investigated an outbr Q fever among 22 out of 57 inhabitants on the outskirts of a l Sheep and cattle were considered to be the main source of infection b was also considered that pigeons might be responsible

Portugal

Fonseca et al[14] showed that the disease exists throughout Portu Up to 1951, 48 human cases had been reported, including 4 laborat infections Positive reactions have been found in 23% of the cows 2 of the goats and 27% of the sheep that have been tested It is conside that the tick *Hyalomma rufipes* is essential for the maintenance of infect among the animal population

Roumania

Combiesco et al[8] reported in 1948 on 3 laboratory infections amor workers investigating a new rickettsial infection in Roumania whic eventually proved to be Q fever

Spain

In 1949, Perez Gallardo et al[27] reported the isolation of *C burneti* from three species of ticks Two of the species, *Hyalomma marginatum* and *Rhipicephalus bursa*, were collected from calves in Seville, the third species, *Rhipicephalus sanguineus*, was collected in Madrid from the dormouse De Prada et al[28] reported upon the first human case of Q fever in Spain in 1950

Sweden

The WHO survey for the period November 1951—April 1952 has yielded negative results from the 1,107 bovine sera examined

Switzerland

Wiesmann[37] states that Q fever is endemic throughout Switzerland and the adjacent territories Between 1947 and 1951, 1,080 human infections were diagnosed at St Gallen and a large number were also found in Zurich The epidemics were traced to cattle, sheep, and goats and to their products A Q fever outbreak in the Engadine valley in 1950 was accompanied by infectious abortions in sheep

Turkey

A survey carried out at Etlik by Minett (personal communication) during the latter part of 1951 showed that of 119 bovine sera, 10 were positive with 3 doubtful reactions, and of 72 sheep sera, 3 were positive with 4 doubtful reactions

bodies before using these animals in their experiments If attempts are made to isolate *C burnetii*, the utmost caution should be used by laboratory workers, since laboratory infections caused by Q fever are very common

Although this second method requires a fairly long time (30 35 days), it has the advantage of the relative ease with which large numbers of animals can be surveyed with little trouble and cost Milk specimens may be collected at the local creameries, and the necessity for bleeding animals is avoided Experience has shown that as many as 5,000 animals can be surveyed in 5 working days one day for collection of milk samples, one day for injection of guinea-pigs, one day for bleeding of guinea-pigs, and one to two days for preparing the sera and performing the complement-fixation test

C burnetii are shed only sporadically in the milk this is true particularly of sheep, and recoveries of the organism from the milk of these animals may consequently be difficult The placentas of infected cows, sheep, and goats are rich sources of *C burnetii* and specimens of this organ can be used, when practicable for the inoculation of guinea pigs

Method 3 Testing of Serum Samples from Individual Animals

This method involves the random selection of blood specimens from individual animals or entire herds in an area Complement fixation tests, as described below, are performed In order to avoid extra effort, blood samples submitted to veterinary laboratories for the brucellosis agglutination test can be used, or blood specimens may be obtained from slaughter-houses

Complement-Fixation Test *

In order to achieve comparable results from the complement fixation tests made in different laboratories, we should like to encourage the utilization of similar techniques in the performance of the test In preparing the following instructions we have tried to take into account variations in the complement fixation technique as performed in laboratories faced with differing diagnostic tasks It is obviously impossible to formulate a technique which will be followed in every detail by all laboratories In [illegible]

laboratories so that future international standardization of these procedures

* The following paragraphs comprise with some additions and amendments the instructions for the performance of the complement fixation test distributed to laboratories collaborating in the Q fever survey sponsored by WHO

antigen is difficult to produce and is expensive to purchase commercially, so any saving in antigen is desirable

In preparing the descriptions of these methods we have consulted workers who have had extensive experience in this field during the past few years In this connexion we should like to acknowledge with great appreciation the wholehearted co operation of Dr Edwin H Lennette of the California Department of Public Health, Berkeley, California, USA Dr Herald R Cox and Mr Don M Wolfe of Lederle Laboratories, Pearl River N Y , USA Dr Robert Huebner of the Laboratory of Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA Dr George Slavin and Dr A W Stableforth of the Weybridge Veterinary Laboratory Surrey, England , Professor E Grasset and Dr J Wirth of the Institute of Hygiene at the University of Geneva, Switzerland and Dr David Lackman of the Rocky Mountain Laboratory Hamilton Montana, USA

Method 1 Examination of Blood Sera of Employees in Fat Rendering Plants

Employees in fat rendering plants have very close contact with practically all types of domestic animals If Q fever exists in the domestic animals of a particular region, it is probable that the sera of workers in the fat rendering plants of that area will give positive reactions in the complement fixation test The presence of Q fever in a country can very well be detected for the first time by this survey method After the disease is known to be present in a region the survey can be extended to specific groups of animals If a Q fever survey is being performed by a veterinary laboratory in a particular country it is urged that co operation of the medical authorities be elicited to obtain the necessary blood samples

Method 2 Survey of Pooled Milk Samples

Milk samples are collected from large numbers of cows, sheep, and goats The raw milk samples of 100-200 animals are mixed together and 3 5 ml are injected subcutaneously or intraperitoneally into 3 5 adult guinea pigs After 30 35 days complement fixation tests are performed on the sera of the injected guinea pigs It should be noted that colonies of guinea pigs are very susceptible to exposure to *C burnetii*, and in laboratories dealing with live *C burnetii* it is essential to isolate in small groups guinea pigs which are inoculated with potentially infectious material (milk, blood, etc) Laboratories working with live *C burnetii* must pre test the sera of experimental guinea pigs for complement fixing anti

This control serum and instructions for its use should be available to national laboratories by the end of 1953

Some laboratories have experienced fading out reactions in the test within one hour after a reading is made, i e a 3+ or 4+ positive reaction read at the completion of water bath incubation becomes + or 2+, or completely negative, after standing for one hour at room temperature This difficulty has been attributed to faulty complement titrations, and if very accurate complement titrations are performed much of this trouble with fading out can be avoided

Interpretation of titre

The majority of positive sheep sera encountered will probably give titration end points of 1/32 or 1/64 while human goat, and cattle sera may give end points considerably higher

In a survey, one is concerned only with the detection of the presence of Q fever in a locality, and the presence of titres of 1/16 in either human or animal sera constitutes presumptive evidence of the presence of infec tion in that particular locality Sporadic single infections, however, are rare in large groups of susceptible domestic animals Thus if upon retest of positive sera selected from the screening procedure, no reactions are encountered higher than at a 1/16 dilution the evidence is not con clusive for past or present Q fever infection in the particular group of animals being tested Usually much higher titres are found in groups of animals where a few end point titres of 1/16 are encountered Where higher titres are encountered all animals in the group which give end point titres of 1/8 or more should be considered as having active or past infection

It should be noted that the actual significance of titres of 1/16 in human beings is unknown From a diagnostic standpoint, in human beings a titre of 1/32 is considered as significant although in a single test such a titre might represent exposure to Q fever months or even years previously In active disease the titre usually rises after 2 to 3 weeks

Serum samples

As far as possible, blood specimens should be taken with the usual sterile precautions, and in the laboratory all serum specimens should be handled with bacteriological technique (sterile pipettes for the removal of serum from the blood clot, chemically clean glass ware, etc) It should be kept in mind that the rickettsial organism may be present in the blood stream and that this constitutes a possible hazard to laboratory and field workers Some difficulties have been encountered with non specific fixing properties which appear in animal sera particularly in the case of sheep, after the sera have been removed from the clot This non specific activity

ill be facilitated It should be noted that the technique given here repre ents, in the main, the one being followed in advanced laboratories, and in all likelihood future variations in this technique made in the interests of standardization will be of a relatively minor nature

Screening procedure

Screening for positive reactions can be done at a dilution of 1/16 of the sera being tested Sera giving a 3+ or 4+ reaction at the 1/16 dilution should be subjected to a second test utilizing serum titrations to 1/128 Undoubtedly certain of the positive sera will give reactions at dilutions greater than 1/128 but for the purposes of survey work and in order to conserve antigen, we suggest titrations only to the 1/128 dilution It is suggested that screening tests be done in groups of at least 20 25 unknown sera in order to limit the use of antigen and positive control serum since a complete titration of positive control serum is required for each group of unknown sera (see tables III and IV)

The serum dilution of 1/16 has been selected for screening purposes because extensive experience has indicated that lower serum dilutions sometimes give non specific fixations Although a non specific antigen control would probably be helpful in testing human sera, especially in countries where typhus fever is endemic or where vaccination against typhus fever is commonly employed, this will not be necessary when testing animal sera, as Q fever antigen is a highly specific product Also, the 1/16 and higher dilutions of sera, which will be accepted as significant reactions in both human and animal sera will eliminate possible non specific reactions which are encountered in lower serum dilutions

Human bovine, or guinea pig sera can be used for positive control in testing homologous or heterologous sera WHO will supply positive control sera on request, but it is suggested that positive control serum be obtained from reacting human beings or animals encountered in a survey In preparing control serum it is preferable to use a pool of several sera instead of a single positive control serum In this connexion, it is advisable to use pools of positive sera with relatively low complement fixing titres i e , 1/32 or 1/64 This is done because low titre sera show greater variation in the fixability of complement than do strongly positive sera, and hence they give a somewhat more accurate idea of the sensitivity of any particular series of tests Also when a low titre control serum is used dilution errors are smaller, and it is easier to obtain identical titres with the control serum in a series of tests The positive control serum is used also to determine antigen units where antigens are prepared locally An international standard anti-Q fever serum is in the process of establishment by the WHO Expert Committee on Biological Standardization

2 *Control positive serum*

Both human and animal control sera should be inactivated in 1/16 dilution immediately prior to the test (60°C for 30 minutes)

3 *Unknown sera*

Dilute 1/16, and inactivate for a total of 30 minutes at 60°C immediately prior to the test

4 *Saline solution*

To 1 litre of 0 85% NaCl solution add 1 0 ml of 10% $MgSO_4$ The presence of magnesium ion appears to sharpen greatly the reactivity of the test In haemolysin titrations, for example much higher titres are obtained when magnesium ion is present than when plain physiological salt solution is used

5 *Sheep red cell suspension*

Fresh defibrinated sheep blood is filtered through several layers of sterile gauze to remove clots Store in the icebox at 4°C Citrated cells have also been found to be very satisfactory For each 50 ml of sheep blood collected use 60 ml of a 3 8% solution of sodium citrate The cells are left in the citrate solution until ready for use Two ml of 1% merthiolate per 100 ml of citrated sheep cells can be used as a preservative The citrated cells should not be used after two weeks storage

A 2% suspension of cells is used for the test Wash the stored cell suspension three times (or until the supernatant fluid is clear) in 0 85% NaCl solution (no Mg ion necessary) by shaking and then by centrifuging the cells for 7 minutes at 2 000 revolutions per minute (r p m) The cells are then packed by centrifugation for 15 minutes at 2,000 r p m A 2% suspension is made by re suspending measured amounts of the sedimented cells in appropriate volumes of 0 85% NaCl If stored in the icebox, the washed cell suspension may be used on 2 successive days, but it is best to make a fresh suspension each day Individual differences in the cells of different sheep, in most cases, will give only slight variations in the titres of haemolysin and complement as determined by the method described

6 *Titration of haemolysin (amboceptor)*

Haemolysin is titrated, as indicated in table I, in the presence of a 1/30 dilution of complement The highest dilution of haemolysin giving complete haemolysis at the end of the indicated incubation period is taken as one unit Some sheep cells are unusually resistant to haemolysis, and because of this it is not advisable to use sheep cells which give a unit lower than 0 25 ml of a 1/1,000 dilution of haemolysin

increases with storage at the usual icebox temperatures It has been observed that animal sera (cattle sheep, and goat) which are stored *on the clot* do not give difficulty as regards non specific reactivity Animal sera may be stored on the clot for 1 2 weeks at 4°C As a rule there is very little haemo lysis during this interval and no bacterial growth occurs If haemolysis does occur, it does not interfere with the test provided that allowance is made, when reading the test for the colour present in the lower dilutions of sera

When serum samples submitted in clotted blood are to be subjected to warm temperatures for more than 24-48 hours (e g during dispatch to testing laboratories) it is best to remove the serum from the clot and if possible to inactivate the serum in a field laboratory at 60°C for 20 minutes Immediately prior to the test in the central laboratory the serum can again be inactivated at 60°C for 10 minutes Sera should be inactivated in the central laboratory *after* they have been diluted 1/16, as this procedure will tend to avoid clotting of certain sera Care should be taken that the water bath should not exceed 60°C, as higher temperatures may destroy antibodies

Reagents and procedure

1 *Antigen*

Dilute the antigen so that 0 25 ml contains 2 antigen units although if the test is carefully done $1\frac{1}{4}$—$1\frac{1}{2}$ antigen units are sufficient The antigen unit should be determined by titrating different dilutions of antigen against corresponding dilutions of a serum of known titre in a checker board titration The highest dilution of antigen which gives a 4+ reaction to the known titre of the serum equals 1 antigen unit

Example

Antigen unknown strength

Serum a control positive serum of known end point titre, in this example it will be 1/64

(Note except for dilutions of antigen and serum the same general method as that described in table IV should be used)

Dilution of antigen	*Dilution of known serum*						
	1/2	1/4	1/8	1/16	1/32	1/64	1/128
1/2	4+	4+	4+	4+	4+	4+	0
1/4	4+	4+	4+	4+	4+	4+	2+
1/8	4+	4+	4+	4+	4+	4+	0
1/16	4+	4+	4+	4+	3+	1+	0
1/32	4+	4+	4+	1+	0	0	0

Result a 1/8 dilution of antigen = 1 antigen unit

Complement is diluted 1/30 with saline and titrated in the presence of 2 units of the antigen as indicated in table II. The smallest quantity of the 1/30 dilution giving complete haemolysis at the end of the final incubation period is taken as 1 exact unit. This unit usually varies between 0.20 ml and 0.25 ml of a 1/30 complement dilution. Very little difference should exist between the complement unit determined in the presence of saline and that determined in the presence of the antigen.

Two exact units, contained in 0.50 ml, are used in the test.

To take an example from table II, suppose that tube 6 is the first tube showing complete haemolysis. Then 0.225 ml of a 1/30 dilution = 1 unit, and 0.450 ml of a 1/30 dilution = 2 units.

To obtain 2 units in 0.5 ml:

$$\frac{0.5}{0.45} = \frac{x}{30}$$

$$0.45x = 15$$

$$x = 33.33$$

Therefore, to 1 part of the original complement serum add 32.33 parts of saline.

The corresponding dilutions necessary for the other tubes are as follows:

Tube number	*Dilution*
1	1/75
2	1/60
3	1/50
4	1/42.86
5	1/37.5
6	1/33.33
7	1/30
8	1/27.27
9	1/25
10	1/23.08

If the end-point falls within tubes 1 to 4 the red cells should be checked for over-fragility; if it falls within tubes 5 to 8 the complement is considered good; and if it falls in tubes 9 or 10 it is preferable not to use this complement as it is weak.

8. *Screening and titration of sera*

Tables III and IV give the procedures used in screening sera at a 1/16 dilution and in the titration of 1/16 positive sera to a 1/128 dilution. It should be noted that in each group of complement fixation tests (screening procedure and titration) it is necessary to run a complete titration of

TABLE I HAEMOLYSIN TITRATION (KOLMER TECHNIQUE)

Reagent*	Tube number									
	1	2	3	4	5	6	7	8	9	10
	Dilution of haemolysin									
	1/1 000	1/2 000	1/3 000	1/4 000	1/5 000	1/6 000	1/8 000	1/10 000	1/12 000	1/16 000
	ml	ml	ml	ml	ml	ml	ml	ml	ml	ml
Haemolysin	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Complement (1/30 dilution)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Saline	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Sheep cells (2% suspension)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
	Incubate for 1 hour at 37 C and then read									

* Add reagents in the order given

Two units of haemolysin are used in all subsequent titrations and diagnostic tests Haemolysin should be titrated at least once a month

As an example for calculation, suppose that, in table I, tube 4 (1/4,000 dilution) is the first tube showing complete haemolysis, this dilution is therefore taken as 1 unit Two units for the complement fixation test would be 0 25 ml of the 1/2,000 dilution of the original haemolysin

7 *Preparation and titration of complement*

Guinea pig complement is dispensed in small quantities in sealed glass ampoules (or tightly stoppered test tubes) It is worth repeating here that all glass-ware and stoppers should be chemically clean as well as sterile If CO_2 refrigerating facilities (— 70°C) are available, the serum is rapidly frozen in a mixture of dry ice and alcohol and stored in the CO_2 icebox Such storage is effective for one month or longer without an appreciable loss in activity Storage is also satisfactory in a deep-freeze unit (— 20°C) or in the freezing compartment of an ordinary refrigerator Freeze dried complement has been used satisfactorily in some laboratories (Certain of the viral and rickettsial antigens particularly the "soluble" antigens, may give non-specific reactions with some batches of guinea-pig complement ... plementary
action
plemen
specific fixation before using the complement in routine diagnostic tests
Q fever antigen is not included in the "soluble" group)

Complement is diluted 1/30 with saline and titrated in the presence of 2 units of the antigen as indicated in table II The smallest quantity of the 1/30 dilution giving complete haemolysis at the end of the final incubation period is taken as 1 exact unit This unit usually varies between 0.20 ml and 0 25 ml of a 1/30 complement dilution Very little difference should exist between the complement unit determined in the presence of saline and that determined in the presence of the antigen

Two exact units contained in 0 50 ml are used in the test

To take an example from table II suppose that tube 6 is the first tube showing complete haemolysis Then *0 225 ml of a 1/30 dilution* = *1 unit*, and 0 450 ml of a 1/30 dilution = 2 units

To obtain 2 units in 0 5 ml

$$\frac{0.5}{0.45} = \frac{x}{30}$$

$$0.45x = 15$$

$$x = 33.33$$

Therefore, to 1 part of the original complement serum add 32 33 parts of saline

The corresponding dilutions necessary for the other tubes are as follows

Tube number	*Dilution*
1	1/75
2	1/60
3	1/50
4	1/42 86
5	1/37 5
6	1/33 33
7	1/30
8	1/27 27
9	1/25
10	1/23 08

If the end point falls within tubes 1 to 4 the red cells should be checked for over fragility, if it falls within tubes 5 to 8 the complement is considered good, and if it falls in tubes 9 or 10 it is preferable not to use this complement as it is weak

8 *Screening and titration of sera*

Tables III and IV give the procedures used in screening sera at a 1/16 dilution and in the titration of 1/16 positive sera to a 1/128 dilution It should be noted that in each group of complement fixation tests (screening procedure and titration) it is necessary to run a complete titration of

TABLE I HAEMOLYSIN TITRATION (KOLMER TECHNIQUE)

Reagent*	Tube number									
	1	2	3	4	5	6	7	8	9	10
	Dilution of haemolysin									
	1/1 000	1/2 000	1/3 000	1/4 000	1/5 000	1/6 000	1/8 000	1/10 000	1/12 000	1/15 000
	ml	*ml*	*ml*	*ml*	*ml*	*ml*	*ml*	*ml*	*ml*	*ml*
Haemolysin	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Complement (1/30 dilution)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Saline	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Sheep cells (2% suspension)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
	Incubate for 1 hour at 37°C and then read									

* *Add reagents in the order given*

Two units of haemolysin are used in all subsequent titrations and diagnostic tests Haemolysin should be titrated at least once a month

As an example for calculation, suppose that, in table I, tube 4 (1/4,000 dilution) is the first tube showing complete haemolysis, this dilution is therefore taken as 1 unit Two units for the complement fixation test would be 0 25 ml of the 1/2,000 dilution of the original haemolysin

7 *Preparation and titration of complement*

Guinea pig complement is dispensed in small quantities in sealed

frozen in a mixture of dry ice and alcohol and stored in the CO_2 [illegible] Such storage is effective for one month or longer without an appreciable loss in activity Storage is also satisfactory in a deep-freeze unit (—20°C) or in the freezing compartment of an ordinary refrigerator Freeze-dried complement has been used satisfactorily in some laboratories (Certain of the viral and rickettsial antigens, particularly the "soluble" antigens, may give non specific reactions with some batches of guinea pig complement These reactions are not believed to be caused by anticomplementary action of the antigens, the reactions have been ascribed to faulty complement In these instances it is necessary to pre test complement for non specific fixation before using the complement in routine diagnostic tests Q fever antigen is not included in the "soluble" group)

Complement is diluted 1/30 with saline and titrated in the presence of 2 units of the antigen as indicated in table II The smallest quantity of the 1/30 dilution giving complete haemolysis at the end of the final incubation period is taken as 1 exact unit This unit usually varies between 0 20 ml and 0 25 ml of a 1/30 complement dilution Very little difference should exist between the complement unit determined in the presence of saline and that determined in the presence of the antigen

Two exact units, contained in 0 50 ml are used in the test

To take an example from table II suppose that tube 6 is the first tube showing complete haemolysis Then 0 225 ml of a 1/30 dilution = 1 unit, and 0 450 ml of a 1/30 dilution = 2 units

To obtain 2 units in 0 5 ml

$$\frac{0.5}{0.45} = \frac{x}{30}$$

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Therefore, to 1 part of the original complement serum add 32 33 parts of saline

The corresponding dilutions necessary for the other tubes are as follows

Tube number	*Dilution*
1	1/75
2	1/60
3	1/50
4	1/42 86
5	1/37 5
6	1/33 33
7	1/30
8	1/27 27
9	1/25
10	1/23 08

If the end point falls within tubes 1 to 4 the red cells should be checked for over fragility, if it falls within tubes 5 to 8 the complement is considered good, and if it falls in tubes 9 or 10 it is preferable not to use this complement as it is weak

8 *Screening and titration of sera*

Tables III and IV give the procedures used in screening sera at a 1/16 dilution and in the titration of 1/16 positive sera to a 1/128 dilution It should be noted that in each group of complement fixation tests (screening procedure and titration) it is necessary to run a complete titration of

TABLE II COMPLEMENT FIXATION (KOLMER TECHNIQUE)

Reagent *	Tube number									
	1	2	3	4	5	6	7	8	9	10
	ml	*ml*	*ml*	*ml*	*ml*	*ml*	*ml*	*ml*	*ml*	*ml*
Complement (1/30 d lution)	0 100	0 125	0 150	0 175	0.200	0.225	0 250	0 275	0 300	0 325
Q fever ant gen (2 units)	0 25	0.25	0.25	0.25	0 25	0 25	0.25	0.25	0 25	0.25
Saline	0 650	0 625	0 600	0 575	0 550	0 525	0 500	0 475	0 450	0 425
	Incubate for 1 hour at 37°C then add									
Haemolysin † (2 un ts)	0 25	0 25	0 25	0.25	0 25	0 25	0 25	0 25	0 25	0.25
Sheep cells † (2% suspension)	0.25	0.25	0.25	0 25	0 25	0 25	0 25	0 25	0.25	0.25
	Incubate for 1 hour more at 37°C and then read									

* Add reagents in the order given

† To save a complete pipetting procedure mix equal port ons of haemolysin and sheep cells *allow to stand at room temperature for 15 minutes and than add 0 50 ml of the mixture to each* tube The mixture should not be used after standing for 2 hours The haemolysin should be poured into the sheep cell suspension when making the mixture

positive control serum (serum A in tables III and IV) and a 1/16 dilution *of known negative homologous serum (serum B) In order to conserve* the antigen needed for a complete titration of positive control serum with each group of tests, as suggested previously, each series of tests should include at least 20 25 serum specimens, for which only one positive control serum titration is required

Table III *outlines the procedure for screening unknown serum specimens* at a 1/16 dilution Table IV describes the procedure for the test itself with the inclusion of serum specimens which have reacted positively at a 1/16 dilution

TABLE III SCREENING PROCEDURE FOR UNKNOWN SERA AT 1/16 DILUTIONS

Serum A (positive control serum)	as in table IV
Serum B (known negative control serum)	*as in table IV*
Serum C (unknown serum 1) Serum D (unknown serum 2) Serum E (unknown serum 3) etc	use only tubes 1 and 5, as indicated in table IV
Antigen Complement Saline Haemolysin Sheep cells	as in table IV

TABLE IV PROCEDURE[a] FOR Q FEVER COMPLEMENT FIXATION TEST AND TITRATION OF SERA POSITIVE AT 1/16 DILUTION

Reagents	Tube number											
	1	2	3	4	5	6[b]	7[c]	8[c]	9[c]	10[c]	11[c]	12[c]
					anticomplementary control for each serum		4-tube complement control (set up for positive control serum (A) only)				haemolytic control	sheep-cell control
	Serum dilution											
	1/16	1/32	1/64	1/128	1/16	1/32						
	ml	ml	ml	ml	ml	ml	ml	ml	ml	ml	ml	ml
Serum: A Positive control serum; B Known negative homologous serum[d]; C Unknown serum 1 (1/16 = positive); D Unknown serum 2 (1/16 = positive); E Unknown serum 3 (1/16 = positive); etc.	0.25	0.25	0.25	0.25	0.25	0.25	0	0	0	0	0	0
Q fever antigen (2 units)	0.25	0.25	0.25	0.25	0	0	0.25	0.25	0.25	0.25	0	0
Complement (2 units/0.50 ml)	0.50	0.50	0.50	0.50	0.50	0.50	0.50 (= 2 units)	0.375 (= 1.5 units)	0.25 (= 1 unit)	0.125 (= 0.5 unit)	0.50	0.50
Saline	0	0	0	0	0.25	0.25	0.25	0.375	0.50	0.625	0.50	0.75
	Incubate overnight at 4-8°C, then add											
Haemolysin[e] (2 units/0.50 ml)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0
Sheep cells[e] (2% suspension)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
	Incubate for 30 minutes at 37°C, then read											

[a] Add reagents in the order indicated.
[b] Tube 6 *may be omitted* if no anticomplementary action of the sera is revealed in tube 5 (*1/16 dilution*) of the screening as outlined in table III.
[c] *Only one set of tubes 7-12 (for the positive control serum) is necessary* for each group of sera being tested.
[d] Tubes 2-4 are not *necessary for* the known negative homologous serum.
[e] To save a complete pipetting procedure, mix equal portions of *haemolysin* and sheep cells, allow to stand at room temperature for 15 minutes and then add 0.50 ml of the mixture to *each tube*. The mixture should not be used after standing for 2 hours. The *haemolysin should be poured* into the sheep-*cell* suspension when making the mixture.

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material is examined on a slide, *Coxiella* can be identified, although this result is not at all easily or constantly obtained

When it can be assumed that the material under examination (e g , blood, ground up organs, urine taken with a catheter, etc) does not contain harmful quantities of other disease-organisms, it can be inoculated directly, if, however, there is a possibility that the material is thus contaminated (as in the case of milk, urine, excrement, etc), it is advisable to mix a certain quantity of penicillin with it (1,000-5,000 Oxford units (OU) or more) This antibiotic has practically no harmful effect on *Coxiella* The same cannot be said of streptomycin, which may inhibit the development of the rickettsiae, especially if the latter are not very virulent or if they are not found in large quantities in the material under examination Chloramphenicol, aureomycin, and oxytetracycline [a] are still more harmful

When the material is very highly contaminated (e g , putrefied material faeces, pus) it should be suitably diluted with normal saline or water and filtered through a Seitz filter (EK discs) If this method is adopted, it should be remembered that *Coxiella* is filterable but that in practice only some of the micro organisms present in the material pass through the filter There-fore, if the material is not very rich in *Coxiella*, a rather large quantity should be filtered and inoculated

Highly contaminated material can be more or less purified by fractional centrifugation Seitz filtration is, however, preferable

The quantity of inoculum in each case varies according to the number of rickettsiae presumed to be present and according to the harmful effect which injection of an excessive quantity of material may have on the in oculated animal When blood is used, 1-2 ml will be sufficient, with milk and urine, up to 3-4 ml can be administered

C burnetii is well known to be resistant both to desiccation and to putrefaction, as well as to many physical and chemical bactericides It can, therefore, be sought with good hope of success even in old, dried, or putrefied material

For this reason, even if there is a certain lapse of time between taking the sample and inoculation, when it is certain that the material under examination has not been contaminated by other organisms and has been handled under sterile conditions, no particular storage precautions need be taken If, however, there is a possibility that the material may be con-taminated, it should be stored at a low temperature to avoid excessive multiplication of the other organisms

It is advisable to keep the inoculated guinea pigs separated from one another during the whole period of observation, so as to avoid transmission

[a] Oxytetracycline is the non proprietary name for Terramycin

of infection It should, however, be mentioned that, with the guinea-pig, transmission of infection from one animal to another is extremely rare, even when healthy guinea pigs live in the same cage with infected ones.

The check for symptoms of infection is carried out in the first instance by the usual method of taking the rectal temperature of the inoculated animals, 40°C (104°F) is considered as the minimum clear sign of fever As a rule, a rise in temperature during the first day after inoculation has no specific significance

Infected guinea-pigs generally show hyperthermia, which continues for one or more days and appears at a more or less early stage, according to the strength of the infective dose If this is strong, the febrile condition may appear even after 24 hours, if it is weak, the fever may not begin for several days or even for several weeks (Burnet & Freeman[8]) When infected blood or milk is injected, the fever generally commences after 7-10 days and continues for 2-4 days

Cases are fairly frequent where infection develops in the animal without any appreciable thermal reaction Consequently, fever in the inoculated guinea-pig has only a relative value and no pathognomonic significance at all—not only because infection may be present without fever but also because many other causes, both infective and otherwise, may produce hyperthermia in the inoculated guinea-pig

A more important test which, however, involves the sacrifice of the animal, is examination of the spleen This should be carried out the day after the fever commences In positive cases, the spleen is found to be very much enlarged, soft, and easily ruptured the surface is greyish and rather rough on account of perisplenitis

Although such findings must be considered as extremely suspicious, they are nevertheless not altogether specific Pathogenic agents other than *C burnetii* can bring about a similar picture It is sometimes possible to identify rickettsiae in Giemsa stained spleen smears, but this is not always easy The rickettsiae are stained a violet-red colour and appear in small groups, either intra- or extra-cellular Care must be taken not to confuse the rickettsiae with the so-called Foà Kurloff bodies, which are normal lymphocytic inclusions in the guinea pig and which sometimes, when stained with Giemsa, take on the aspect of small masses of granules or minute violet red rods

The only certain proof of infection in the guinea-pig is the detection of the specific antibodies in the blood of the animal The antibodies may appear in as short a time as the 7th to 9th day, but when the infective dose is not very large they appear much later If quick results are required, therefore, serodiagnosis may be carried out as early as 10 days after inoculation with the suspect material If a negative result is obtained ·

is advisable to repeat the serodiagnosis 30-40 days after inoculation The appearance of the antibodies is particularly late when milk injections are used, since the milk sometimes contains only very small numbers of rickettsiae

The antibody titre in infected animals is very high more than 1/32 1/64

A low titre will sometimes be obtained if the inoculated material contains a large quantity of dead rickettsiae It should be remembered, as observed by Babudieri & Secchi,[1] that in some rare cases the presence of pyogenic abscesses in the guinea pig may cause the appearance of a non specific complement fixation reaction at a low titre (up to 1/16) This happens fairly frequently after subcutaneous injection of milk samples

In such cases the agglutination test remains negative, and deviation of the complement becomes negative as soon as the abscess empties or heals In doubtful cases another animal may be inoculated with a sample of the spleen of the positive animal the antibodies can then be sought in the inoculated animal

The suspect material can be injected not only into the guinea pig but also into the mouse, rabbit, or hamster The mouse reacts well to the inoculation and it is relatively easy to trace *Coxiella* in its spleen There are certain disadvantages in using the mouse, however, for example it is not possible to use large doses of the infected material, the animals die easily from infection caused by organisms present in the inoculum, and they are easily infected when in contact with, or even when near, *Coxiella* infected mice

The rabbit is less sensitive to the infection than the guinea pig and is only used in special cases—in particular for direct detection of rickettsiae in the necrotic tissue after injection of the infected material

The hamster is very sensitive to the infection but has no particular advantage over the guinea pig which is more often available in laboratories

In special cases and when it is quite certain that the material has not been contaminated the vitelline sac of the chick embryo can be inoculated on the 7th day of development Seven days from the date of inoculation, smears of the vitelline membrane stained with Giemsa or Macchiavello are examined for *Coxiella*

This method is only rarely used, since it calls for special equipment and technique and does not often give positive results In fact, *C burnetii* cannot be easily adapted to growth in the chick embryo

Finally, it may be recalled that Burgdorfer[2] has proposed demonstrating the presence of *C burnetii* in the blood by means of a tick—*Ornithodorus moubata* The tick is applied to the skin of the patient and left

there until it is well filled with blood After 20 days it is killed, and smears from the intestine are examined for rickettsiae

Obviously, this method of research has disadvantages and can be applied only in very special cases

Serological Tests

Complement fixation

The complement-fixation test is the most widely-used serological test for the diagnosis of Q fever

It can be carried out with antigens prepared in the laboratory or with commercial antigens The latter are preferable since the preparation of a good antigen is very complicated and not always successful, and is, moreover, extremely dangerous on account of the very high infectivity of *C burnetii* cultured on the chick embryo

Antigen

If it is desired to prepare an antigen suitable for the complement-fixation test, a strain of *C burnetii* must be used which develops easily and abundantly in the chick embryo The "Henzerling", "Nine Mile", "Zurich", and "Grottazzolina" strains may be mentioned as being among those which are most frequently used for this purpose

Hens' eggs which have been incubated for seven days at 38°C are used The eggs are inoculated in the vitelline sac with 0 2 ml of a suspension of *C burnetii* infected vitelline sac in physiological solution The suspension is prepared so that the infective dose causes the death of about 30% of the embryos on the 7th day after inoculation In general, such a dose is obtained by diluting a sac very rich in *Coxiella* in 1,000 2,000 ml of physiological solution It is advisable to add 200-500 OU of penicillin per ml of suspension Embryos which die within the first four days after inoculation are eliminated From the fifth day, non-viable eggs are opened with aseptic precautions, and the vitelline membrane is extracted and placed in a Petri dish A smear, which is fixed with methanol and stained with Giemsa, and a sterility control culture in broth and in agar, are prepared from each membrane

On the seventh day all the eggs, including the viable ones, are opened, and controls are prepared from all of them The sacs which are found to be contaminated or to contain only a few rickettsiae are eliminated, the others are collected and kept in the refrigerator

One hundred non-contaminated sacs rich in coxiellae are emulsified by means of a "Turmix", with the addition of physiological solution to which 0 5% of formalin has been added A good emulsion is generally

obtained in three minutes The emulsion is stored in the refrigerator for 24 hours Subsequently, after adjusting the pH of the suspension with normal hydrochloric acid to 5 5-5 8, it is centrifuged for one hour at 5,000 6,000 revolutions per minute (r p m) The precipitate is taken up and re suspended, with the aid of a " Turmix ", in 100 ml of non formolized physiological solution

The suspension is mixed with 200 ml of chemically pure ether and, after vigorous shaking, is transferred to a separating funnel and kept in the refrigerator overnight On the following day, the suspension will be found to be in three layers—a top layer of ether, a middle layer of a whitish, opaque suspension rich in vitelline sac elements, and a lower greyish opalescent layer This last layer, which contains the greater part of the rickettsiae, is collected and placed in the refrigerator To the rest of the suspension 100 ml of physiological solution are added, and the mixture is shaken and left in the refrigerator for three hours The lower layer is then again separated and added to that collected previously If necessary, the addition of physiological solution to the rest of the mixture can be again repeated The ether is removed by means of a vacuum pump from the suspension of coxiellae thus obtained, and the suspension is centrifuged for one hour at 5,000 6 000 r p m The precipitate is once more extracted with ether and then taken up in physiological solution and again centrifuged Finally, the precipitate is collected in 50 ml of physiological solution (1 ml per two sacs) and agitated in an ultrasonic oscillator for the time necessary to obtain a good distribution of the rickettsiae (microscopic control)

At this point it is advisable to irradiate the antigen, poured in a thin stream, with ultra-violet rays (e g , Hanau NK 25/85 5 watt lamp at a distance of 20 cm, for 4 minutes) in order to kill any rickettsiae which may have resisted the action of the formol The antigen is stored in the refrigerator but freezing must be avoided as this reduces its potency Before the antigen is used, it must be controlled for specificity, sensitivity, and absence of anticomplementary power This control is effected as follows Using a standard antigen with a definitely negative human serum, the amboceptor and complement are titrated for use as indicated below Then, in geometrical progression, dilutions of the antigen under examination are prepared, ranging from 1/4 to 1/64, using normal saline solution or, better still, a solution containing 0 85% NaCl and 0 01% $MgSO_4$

Three series of Wassermann tubes are then prepared and to each is added 0 2 ml of the various antigen dilutions

[illegible] negative

[illegible] cond

[illegible] ded,

and third series, equal quantities [illegible]

diluted to the limiting titre and to half the limiting titre respectively Finally, 0 2 ml of the previously determined complement dilution is added to all the tubes which, after brief shaking, are kept for one hour at 37°C in a thermostat or, preferably, in a water bath 0 4 ml of the haemolytic mixture (amboceptor and suspension of sheep erythrocytes) is then added to each tube, and the tubes are maintained at 37°C for another hour, when the reaction is read

The dilution for the final test is fixed at half the maximum dilution giving a definitely positive result with the positive serum at its limiting titre (2 units of antigen) For example, if the maximum dilution giving a positive reaction is 1/32, a 1/16 dilution will be used for serodiagnosis

The antigen can be employed when the test carried out with the negative serum has shown it to possess anticomplementary activity at a dilution not greater than one-sixth of the maximum, as determined by the preceding test (1/5 3 in the previous example) A greater anticomplementary power can be tolerated on condition that it never exceeds one quarter of the maximum titre of the antigen Its value and anticomplementary activity can be more exactly established in a subsequent test, using antigen dilutions nearer to the limiting titre

Technique of test

This technique is described in the preceding paper by Kaplan & Hulse (see page 175)

Agglutination

Antigen

The antigen for the agglutination test is prepared more or less by the same method as that for the complement fixation test (see page 197) It must, however, have a higher degree of purity, and this is obtained by repeating the ether extractions and centrifugation in order to eliminate any heterogeneous elements There must also be perfect dispersion of the coxiellae, there must be no clumping, however small, of the micro-organisms This can be ensured by subjecting the antigen to ultrasonic treatment for a longer time 0 01% of merthiolate is added as an antiseptic.

The dilution of the antigen is based on test samples, and varies according to the technique to be employed for the agglutination For the microscopic method it is desirable to have a dilution ten times greater than that used for the macroscopic method

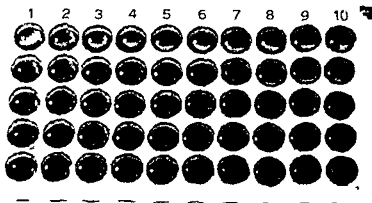
Serum under examination

The serum must be perfectly clear, otherwise it should be centrifuged It must be inactivated at 58°C for 30 minutes Fresh serum contains an anti-agglutination factor

Microscopic agglutination

Microscopic slide agglutination is the most economical serological test for the diagnosis of Q fever For this, however, special slides—made of thick glass or crystal with 50 circular areas of 10-mm diameter, arranged in 10 rows, numbered, and separated by an unpolished glass surface—are necessary (see fig 1)

FIG 1 SLIDE FOR MICROSCOPIC AGGLUTINATION TEST

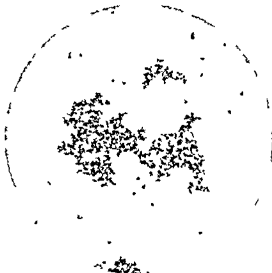


Using a 0 1 ml pipette graduated in hundredths, 0 02 ml of the antigen is placed in each depression Separately, five tubes are prepared with dilutions of the serum graduated in geometrical progression, from 1/4 to 1/64 For the dilution, use physiological solution containing 0 01% of merthiolate Using another pipette graduated in hundredths, place 0 02 ml of the various serum dilutions in the five depressions of each row *The same pipette can be used for the various dilutions of the same serum* as long as one begins with the highest dilution

With each group of agglutination tests, controls are carried out, using a definitely positive serum diluted to *half its titre*, and a definitely negative serum diluted to 1/4 and to 1/8

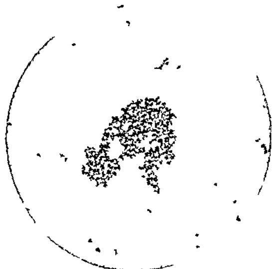
The serum and antigen in each circular area are mixed, and the mixture is spread over the whole surface, using a platinum needle *sterilized in flame and allowed to cool* Finally, the slide is placed in a damp chamber (a large Petri dish containing a wad of cotton wool soaked in water will serve) and left at room temperature throughout the night On the following day *the slide is removed from the Petri dish* and, without shaking it, is left to dry in a thermostat at 37°C It is essential that drying take place slowly

FIG 2 AGGLUTINATION OF COXIELLA BURNETII



Elements showing b po a sta n ng can be seen magn f cat on 700 x

FIG 3 AGGLUTINATION OF A CONTAMINATING MICRO ORGANISM MORPHOLOGICALLY VERY SIMILAR TO COXIELLA BURNETII



No elements showing bipolar staining can be seen The bacilli are most intensely stained and stand out more clearly Magn fication 700 x

FIG 4 AGGLUTINATION OF COXIELLA BURNETII WITH INCREASING SERUM DILUTIONS



Magnification 180 x

FIG 4 AGGLUTINATION OF COXIELLA BURNETII WITH INCREASING SERUM DILUTIONS (con nued)





n at on 180 x

Methanol is then poured over the surface of the slide, which is left for 3 minutes The preparation is then stained, preferably with Giemsa (one drop per ml of distilled water, for 25 minutes) or alternatively with a buffered methylene blue solution (0 65 g of methylene blue dissolved in 25 ml of distilled water containing 2 5 g of acid sodium phosphate The solution is heated at 100°C for 6 minutes, after which 3 125 g of potassium dihydrogen phosphate are added and the total volume of the solution is made up to 250 ml)

The slide is then washed in running water and left to dry It is examined with a dry objective enlarging 100-200 diameters, after clearing the preparation with a drop of cedar oil The immersion objective is used only in doubtful cases or for purposes of control (see fig 2, 3)

If the test is negative, the coxiellae remain almost colourless and are distributed in a uniform manner over the whole field, with a certain tendency to gather more thickly and regularly around the edge If the test is positive, they concentrate in the centre of the field, forming more or less large, irregular clumps which are intensely stained with Giemsa or methylene blue (see fig 4)

Sometimes, especially when the serum is strongly haemolysed, examination of the 1/4 dilution is somewhat difficult, at a 1/8 dilution reading becomes easier

In some rare cases a paradoxical phenomenon is observed, whereby agglutination is absent in the first, and even in the first two, dilutions, and becomes evident only from the third

Macroscopic agglutination

[illegible] more easily carried out It is, [illegible] nption of antigen, which is [illegible] ployed for microscopic agglutination

In this test, 0 2 ml of graduated dilutions of the serum under examination, and 0 2 ml of the antigen, are placed in a series of agglutination tubes The tubes are left at room temperature for 24 hours

Agglutination begins after one hour with the appearance of small granules which tend to fall to the bottom After 24 hours the liquid is perfectly clear and the bottom of the tube is covered with a kind of thin film, which breaks into small fragments as soon as the tube is shaken (see fig 5 7)

Macroscopic agglutination can also be carried out on the slide, particularly as a rapid preliminary test For this, use a hanging-drop slide and in the hollow mix 0 04 ml of antigen with 0 04 ml of serum in a 1/4 to 1/10 dilution Move the slide slowly to and fro so as to encourage agglutination, which becomes evident after 20-30 minutes

FIG 5
MACROSCOPIC TUBE AGGLUTINATION NEGATIVE REACTION

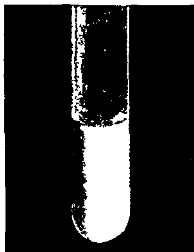
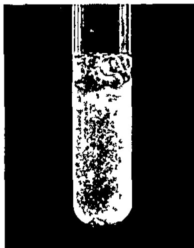


FIG 6 MACROSCOPIC TUBE AGGLUTINATION POSITIVE REACTION (BEFORE SHAKING)



FIG 7
MACROSCOPIC TUBE AGGLUTINATION POSITIVE REACTION (AFTER SHAKING)



Agglutination with dried blood drops

In special cases, particularly in large scale research for epidemiological purposes, it may be useful to perform the agglutination test using dried blood-drops For this purpose, collect a drop of the blood under examination with an ordinary platinum loop, place it on a slide, and leave it to dry Blood sampled in this way, with a volume of 0 03 ml corresponds to about 0 02 ml of serum

In order to carry out the test, add to the dried blood drop 0 02 ml of physiological solution containing 0 01% of merthiolate Graduated dilutions of the drop can then be made To each drop of diluted blood add next an equal volume of antigen, and carry out the test as described for microscopic agglutination

Since the blood may take too long to dry on a slide, particularly in winter or in a damp atmosphere, a thin numbered strip of filter paper, protected by two strips of cellophane, may be used to absorb the drop When carrying out the test, cut off the paper impregnated with the drop of blood and let it soak in a test tube containing the quantity of physiological solution necessary to obtain the required dilution Agglutination is then obtained with the technique described above

The dried blood agglutination test does not give exact quantitative results, but it is particularly convenient when preliminary tests have to be performed on a great number of people The reaction can be obtained even with blood which has remained desiccated for two months

Opsonin Test

This test has recently been advocated by Victor et al [4] It is described as being extremely sensitive, but it has not yet been sufficiently tried out in practice and sometimes gives results which are difficult to interpret

Antigen

The antigen used is *Coxiella* cultured on vitelline sac, killed by treating with 0 5% phenol for 7 days at 5°C, and subsequently washed repeatedly with physiological solution and concentrated by centrifuging at 20 000 r p m The coxiellae are then diluted empirically to the dilution giving the best results

Technique

0 1 ml of the heparinized blood of the person under examination is mixed with 0 1 ml of antigen, in a test tube Plasma or serum can be used instead of heparinized blood In this case washed leukocytes

of another, normal, person must be added to the mixture The mixture is agitated by rotation at 37°C for 30 minutes Smears of the mixture are then prepared on slides and stained by the Macchiavello method (After fixing by flame, stain for 5 minutes with 0.25% basic fuchsin solution, wash in running water, and decolorize the preparation for 20 seconds in two stages with 0 5% citric acid solution Wash the slide, and re stain for 30 seconds with 1% methylene blue solution Wash in running water and leave to dry) Examine under the microscope

It is necessary to determine the number of neutrophil leukocytes which contain phagocytized coxiellae in their protoplasm The reaction is considered as positive if 94% of the neutrophils have phagocytized the rickettsiae

A unit of opsonin is considered as the quantity capable of bringing about phagocytosis with 94% of the neutrophil leukocytes The opsonic titre is given by the number of units contained in 0 1 ml of the serum or plasma of the person under examination

[illegible]

dil [illegible]

this [illegible]

solution, 4 parts of 1 15% KCl 3 parts of 1 22% $CaCl_2$, 1 part of 2 11% KH_2PO_4, 1 part of 3 82% $MgSO_4$ $7H_2O$, 4 6 parts of 5% $NaHCO_3$

This same solution is used for washing the leukocytes

The opsonins keep for a fairly long time in serum stored at + 5°C

Allergy Tests

Palpebral test

This reaction can be used only in animals especially ovines and bovines

Antigen Begin with a suspension of *Coxiella* infected vitelline sac, [illegible]

an hour at 6,000 r p m and retain the sediment only Bring this to the original volume by the addition of formolized physiological solution, and centrifuge for 5 minutes at 2 000 r p m

Take up the sediment in 1 ml of formolized physiological solution per vitelline sac, and re centrifuge for 5 minutes at 2 000 r p m Add the supernatant liquid to that collected after the previous centrifugation and centrifuge again for 5 minutes at 2 000 r p m Then discard the sediment and centrifuge the supernatant liquid for an hour at 6 000 r p m The sediment thus obtained is suspended in 4% fo[illegible]

solution (10 ml per vitelline sac), and constitutes the final antigen Before use, it is necessary to ascertain not only that this antigen is free from other organisms but also that it does not contain any live coxiellae To confirm this, either a sterility control test is carried out on the usual media, or some guinea pigs are inoculated with 0 5 ml of the antigen, kept under observation for a sufficient time, and then examined serologically (It should be noted that injection of this considerable quantity of dead coxiellae provokes the appearance of antibodies in the guinea pig, but at a low titre only)

The ordinary antigen used for the complement fixation test can also be used for the palpebral reaction

Technique After rapid disinfection of the skin, inject the antigen subcutaneously in the lower eyelid of the animal under examination

In the case of bovines and horses inject 2 ml of antigen, with sheep, 1 ml If instead of the special antigen, the complement fixation antigen is used, the doses should be respectively 3/8 and 2/10 ml

A positive reaction becomes apparent after three to four days, with intense swelling and inflammation of the palpebra which reaches its peak about the fifth day and persists for several days

In positive cases the palpebral reaction causes a short bout of fever and a considerable although not very persistent (40-50 days), increase in the serum antibodies

Intradermal test

The intradermal test has been used in man but with not very convincing results since quite a number of healthy persons react non specifically but quite strongly to the injection of the antigen On the other hand the test gives good results in the guinea pig

Antigen The antigen is the same as those used in the palpebral test

Technique Inoculate with a fine needle within the shaved skin of the abdominal region of the guinea pig

The antigen dose is 0 1 ml if the complement fixation antigen is used, it should be in a 1/1 dilution with physiological solution

In positive cases a more or less extensive infiltration appears at the site of inoculation, together with a hardening of the tissues which can be felt on palpation This reaction appears only after 5 7 days and some times later, and persists for several days

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2 Burgdorfer, W (1951) *Acta trop , Basel* 8 44

3 Burnet, F M & Freeman, M (1937) *Med J Aust* 2 299

4 Victor, J , Raymond R , Valliant J Wagner J C & Pollack A D (1952) *J exp Med* 95, 61

DISCUSSION: PART IV

Some of the problems connected with the laboratory diagnosis of Q fever were raised Several participants stressed the need for international standards for specific Q fever sera, and for agreement on antigen concentration It was pointed out that WHO would soon establish an international standard anti Q fever serum for use in the complement fixation test This would serve to standardize the principal diagnostic serological [illegible]

The value of the different diagnostic laboratory tests was discussed It was pointed out that in viewing this problem the time for development of specific antibodies had to be considered The agglutinin antibodies seem to develop at a slightly later stage than the complement fixation antibodies, but the former seem to persist for a longer period The complement fixing antibodies may develop slowly, and in some cases before they can be demonstrated more than two weeks may elapse after symptoms appear In human cases treated with antibiotics a serological diagnosis may not be made, at times, until 30 days after the onset of the disease The value of the agglutination test was stressed although this test requires a greater quantity of antigen than does the complement fixation test

Intracutaneous allergic testing in sheep and goats was also discussed It was stated that this test can give rise to considerable specific antibodies in the recipient 0 1 0 2 ml of antigen could give titres amounting to 1/256 and remaining at a high level for months

The problem of differential diagnosis in man from diseases such as psittacosis, typhus, and influenza was raised It was stated that little difficulty should be encountered after one to two weeks, since serological tests for Q fever are highly specific, especially where a rising blood titre is encountered

Variations in the character and transmission of the disease in different areas were discussed In Italy, for example, in some of the central areas the disease tends to appear regularly at the time of parturition of small ruminants, in the islands the disease occurs all the year round In the USA a frequent channel of infection seems to be the alimentary tract while in Europe the inhalation route seems to predominate

As regards the effect of Q fever in animals, it seems, on the whole, that no clinical sign of disease is apparent Pneumonia and abortion among small ruminants have been reported, but further confirmation is needed on this point *Coxiella* has been isolated many times from the placenta of cows, sheep, and goats without apparently causing illness or abortion This fact undoubtedly plays an important part in the epidemiology of the disease

The results of vaccination against Q fever in humans were discussed This procedure is not entirely without danger as certain toxic effects resulting in a nephritis have been encountered Vaccination of highly exposed groups, such as laboratory personnel, has been successfully carried out in the USA, for the present, it appears advisable to restrict vaccination to such groups

The question was raised as to whether wild animals, particularly rodents, could act as a reservoir for Q fever since in many countries ticks are known to be transmitters of *Coxiella* It was felt that this aspect of Q fever was worth investigating perhaps results from such investigations would shed light on one of the several obscure epidemiological facets of the disease

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Part V

RABIES

RABIES

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Chief Virus Section
Institut Pasteur Paris France

Rabies is a disease of mammals caused by a specific virus transmitted through the bite of rabid animals to other animals and to human beings, resulting in rapidly fatal encephalomyelitis after a more or less lengthy incubation period

THE DISEASE

Natural Infection (Street Rabies)

Canines

In 86% of cases, it is the dog [illegible]
wild carnivores 3 5%, ruminants [illegible]
an incubation period of 15 to 90 d[illegible]
marked by a modification in the [illegible]
the animal, the initial symptoms rarely noted, are high fever and pupillary dilation The disease may then develop in one of two forms

(*a*) *the furious form* with excitement, aggressive fits of dementia, bolting, rabid barking, and terminal paralysis or

(*b*) *the paralytic form* (dumb rabies) from the outset, beginning either with the hindquarters or with the jaws and extending more or less rapidly (over 1 to 7 days) to the respiratory centres

While this is a convenient differentiation which agrees, by and large, with the facts, it must be kept in mind that only mixed types of infection exist, in which either excitation or paralysis predominates, and that all forms end in a paralytic period

It should be noted that

(1) the virulence of the saliva often begins 48 hours (and exceptionally up to 14 days) before the first symptoms of rabies,

(2) the existence of a terminal glycosuria is a good diagnostic symptom,

(3) the symptom of hydrophobia never appears in the dog, which continues to seek food and drink until the terminal phase

Cats and wild carnivores

Rabies usually appears in the furious form, the severity of the bite of such animals is due to the depth, the site (on the face), and the nature of the saliva of carnivores in which hyaluronidase is a diffusion factor Wolves are responsible for 0 1% of all bites, which, in spite of treatment, result in 6 8% of the total deaths from rabies

Herbivora, birds, and hibernators

Rabies is always of the paralytic type with a long incubation period In the case of the marmot, the incubation period is suspended during hibernation

Cheiroptera

There has long been observed in Latin America—particularly in Argentina, Brazil, Mexico, Paraguay, Trinidad, and Venezuela—an epizootic paralysis of cattle, characterized by a sudden posterior paraplegia (*mal de Caderas*, derriengue, etc) occasionally transmitted to man in the form of myel[illegible] ntained and inocu- [illegible] which the *infection* [illegible]

Experimental Rabies

Laboratory animals

The rabbit is the classic laboratory animal Its constant susceptibility, which serves as a standard of comparison of strains, and its consistently paralytic manifestations, even with the street virus, make it the animal of choice for experimental work

The guinea-pig is more susceptible than the rabbit, it is often used for the diagnosis of suspected material, but it sometimes shows symptoms of nervous excitement

The mouse, sensitive to various routes of inoculation whatever its species, has become the most widely used animal because of its low cost, its easiness to handle, and, above all, its short rabies incubation period

The hamster, as susceptible as the mouse and more consistent in its manifestations, is a valuable animal, particularly for serum neutralization and vaccination tests

Plurality and diversity of strains

The strains of rabies virus encountered in the natural or street virus differ considerably from each other in such characters as degree of virulence,

length of incubation, intensity and type of histological lesions, and antigenic strength Nevertheless, together they form a complete antigenic community, whatever their origin, from the highly virulent viruses to the African strains of oulou fato whose invasive power is so attenuated that it is rarely pathogenic for man

This antigenic unity does not answer the question whether it is better to vaccinate individuals with local strains or even to prepare autogenous vaccines, *for instance, from the brain of the biting dog, rather than with* strains of fixed virus of controlled antigenic value

Adaptation and fixation of strains

Pasteur showed that repeated passages of the street virus in the same species of animal, in particular the rabbit resulted ultimately, after a number of passages varying according to the strain but averaging 40 to 80 in serious and irreversible modifications in the behaviour of the strain, which was transformed from a street virus into a fixed virus

Fixation is characterized by

(*a*) the constancy of the incubation period, which shortens progressively to a minimum of 4 to 9 days according to the strain and which remains unchangeable thereafter,

(*b*) the regularity of the symptomatology in animals and the exclusively paralytic form of the disease

(*c*) the higher degree of cerebral virulence and its constancy once the virus is fixed,

(*d*) histologically, the disappearance of Negri bodies and their replacement by nuclear lesions of a particular type

As a matter of fact, a careful study of fixed strains carried out over a period of several years shows that the evolution of the virus, once fixed, continues at a very slow though progressive rate,[15] as shown by the gradual rise in the degree of cerebral virulence paralleled by a reduction in the affinity of the virus for the various parts of the peripheral nervous system It is therefore necessary for all laboratories to check their fixed viruses periodically or to use only strains of known characteristics

The majority of antirabies institutes use the fixed virus strain, known as the Pasteur strain, which was used in the first vaccinations, and especially the Paris sample of this strain, which has been maintained exclusively in rabbits since its origin at the Institut Pasteur in Paris The reason for this choice is that this strain is still the one which provides the most satisfactory immunizing power and which is the most harmless

PATHOLOGY

Only microscopic examination of the central nervous system (brain, medulla oblongata, and neuroganglia) has absolute value. It shows, first, the presumptive indications of rabies · encephalitic and neuroganglial lesions. Secondly, there are the definite indications. specific cellular lesions

Encephalitic lesions

These are the lesions common to all types of encephalitis. They are

(*a*) *encephalitic foci*, that is Babes's nodules (1892), accumulations of infiltration and neuronophagia localized in any part of the parenchyma, but particularly in the neighbourhood of the central nuclei and in the subcortical regions,

(*b*) *perivascular sleeves*, surrounding the capillaries and the small vessels, becoming more marked the longer the disease persists,

(*c*) *meningitis*, sometimes intense, predominating at the base of and along the principal septa

Ganglial lesions[8, 9]

These are easily observable on the large neuroganglia, such as the [illegible] and the ganglion nodosum they are marked by cellular [illegible] of which [illegible]

Specific lesions

(*a*) *Street rabies* (see fig 1 and 2) Negri bodies,[18] round or oval in shape, appear as inclusions measuring 0 25μ-20μ in diameter and situated in the cytoplasm of otherwise normal neurons Special staining * is necessary to see these lesions They have a predilection for the internal or middle layers of the Ammon's horn as well as for certain grey nuclei (for example, of the basal optic ganglion) and for the pyramidal cells of the hippocampal surface At times, two or three may be found in the same neuron, often at the base of the axon The proportion of affected cells is very variable, usually in the neighbourhood of 20%, but often much less

(*b*) *Fixed virus rabies* (see fig 3) Oxyphilic nuclear lesions[16] occur in the nerve cell, which is considerably altered and pyknotic. Rounded or oval corpuscles of irregular size may be seen in an acidophilic mass

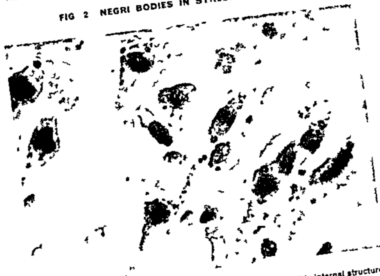
* See annexes to article by Lépine on methods of rabies diagnosis page 239

FIG 1 NEGRI BODIES IN STREET RABIES I



Strain produc ng la ge Neg bod es w th v s b e nte nal structure
Mouse Ammon s horn

FIG 2 NEGRI BODIES IN STREET RABIES II



Stra n produc ng very numerous small Negri bod es without vis ble internal structure
Mouse Ammon s horn

FIG 3 TYPICAL POLYCHROMATIC AND POLYMORPHIC NUCLEAR LESIONS IN FIXED VIRUS RABIES*



* Pasteu st a n
Rabb t Ammon s ho n

representing the remains of the nucleus these corpuscles are hyperchromatic and are mainly basophilic though some are strongly acidophil c

The specific lesions mentioned under (*a*) and (*b*) above are always found together but in a varying proportion with a strong predominance of one or the other according to whether the street or the fixed virus is involved It is possible to diagnose strains of street virus especially those with short incubation periods where Negri bodies are rare or absent when these lesions are recognized

VIRUS CHARACTERISTICS

The rabies virus is strictly neurotropic it is invisible and filterable and about 150 μ in size It spreads along the nerves to the pyramidal cells and ganglia of the nerve centres where it mult plies It is not found in the blood or in secretions of urine bile or milk It is very resistant to cold desiccation and glycerol and somewhat resistant to alcohol ether and acetone It is destroyed by heating for 15 minutes at 50°C for 5 minutes at 60°C or for 2 minutes at 100°C It is inactivated by ultra violet rays proteolytic enzymes and antiseptics such as phenol and formol

represent at present more than nine tenths of the antirabies vaccines used in the world The most widely-used form of this type of vaccine is a 5% suspension of sheep brain inoculated with fixed virus, phenolized to 1%, and attenuated for 24 hours at a temperature of 37°C Innumerable variations in procedure exist, depending on what species of animal (for instance, sheep, rabbit, monkey, or dog) supplies the virus, on the temperature and duration of the attenuation period (37°C, 22°C, etc), on the concentration of the vaccine (5%-20%), on the percentage of phenol (reduced in the USA to 0 25%), on the volume injected (0 5-5 ml per injection), and on the number of injections (7-30) necessary for treatment

Physical inactivation

The standard is represented by the vaccine which is inactivated by means of ultra violet rays A virulent emulsion is exposed to ultra violet rays for a period which varies according to the type of apparatus used Merthiolate is then added (1/10,000) to inactivate the proteolytic enzymes which have not been affected by irradiation, and which would rapidly destroy the antigenic power of the vaccine The results obtained with irradiated vaccines have on the whole been excellent

Living avianized vaccines

To the two types of vaccine mentioned above which, with their variants, comprise almost the entire range now employed in man, there must be added a third type, which has recently appeared and which has as yet been employed only in animals—the living avianized vaccines

Strains of street virus have been egg adapted [13] After a variable number of passages in eggs, they have lost the power to cause rabies, while retaining their antigenic power and producing in the animal into which they are injected *intramuscularly* an active immunity which is more certain in its effect than that produced by killed vaccines Vaccination with avianized vaccines has amply proved its worth, especially for the vaccination of dogs Its application to man however, is still in the experimental stage

Antirabies Treatment of Humans

Indications for antirabies treatment

Vaccination of humans *is given after* infection The seriousness of rabies makes it necessary to consider all bites by unknown animals or animals behaving abnormally, whether wild or domestic, to be suspect The course to be followed after a person has been bitten will depend upon

whether the biting animal can be kept under observation in no case should it be killed The procedure is as follows

(*a*) If the animal is alive and appears to be in good health, it should be placed in quarantine and examined by a veterinarian If it remains alive after 15 days, no treatment is required If the bites are on the face, treatment should be discontinued on the eighth day if the animal is still alive

(*b*) If the animal has been killed or has disappeared, treatment is necessary However, if the brain of the killed animal shows no lesions and the bites are not serious, the results of mouse inoculation with a cerebral emulsion obtained from the biting animal may be awaited

(*c*) If the animal is rabid, or if examination of the brain shows even non specific lesions, treatment is necessary

(*d*) If the bites have not broken the skin or if there has been licking or contamination with saliva which has not reached the mucosa, no treatment is required However, soiling of the buccal, nasal, or other mucosa by a known rabid animal renders treatment imperative

Incidents, accidents, and failures in antirabies treatment

In 5% of patients treated incidents of a non serious nature occur, taking the form of local erythematous reactions arising between the sixth and the tenth injection and diminishing thereafter these are rarely accompanied by a general reaction, which is seen mainly in liverish or obese persons

Paralytic accidents sometimes occur towards the end of or after treatment, with a frequency (1 in 700 in Los Angeles, USA. to 1 in 15,000, average 1 in 10,000) varying according to the vaccines and methods employed These are most often in the form of flaccid paraplegia or Landry s paralysis, which often regress and recover without sequelae after several days sometimes the disease develops fatally or leaves residual paralysis after cure A study of accidents [20 22] shows that they have several possible causes, such as a fixed virus, a toxic and demyelinating action of neuroproteins, or sensitization to a latent virus, and that they are no more frequent with intensive treatment than with reduced dosage treatment

Finally, all antirabies institutes see a certain number of failures, that is, cases of rabies that have developed in spite of regular treatment Some are due to viruses with short incubation periods which become manifest before the end of the 20-day period normally considered sufficient for the establishment of immunity, many institutes omit these cases from their statistics, considering them incurable Other failures occur sooner or

[illegible] normal treatment, these are considered as [illegible]

[illegible] has paradoxically, caused some doubt [illegible] even though there is ample statistical [illegible] of the protection conferred on man by antirabies [illegible] However, these failures should not lead the institutes to [illegible] for unusual mortality All institutes in which the [illegible] is greater than 0 1% of treated cases or in which [illegible] after the [illegible] day are as numerous as deaths before that date [illegible] their technique faulty and their treatment insufficient

[illegible]

(a) Local treatment of bites, if practised immediately, reduces the chances of infection Deep washing with 20% concentrated liquid soap has been shown to be the most effective form[25] and is better than the classic cauterization with fuming nitric acid Sulfonamides and antibiotics have no effect on the virus but act on secondary infections

(b) There is no benign rabies but only a greater or lesser chance of contracting rabies. If therefore it is decided to treat a bitten person the treatment given must be normal and complete Shortened and insufficient treatment leads only to false security and failures

(c) *In countries where rabies is endemic or in periods of endemicity the risk of contracting rabies overrides all other considerations, possible accidents are much more rare than rabies itself and are not more frequent after intensive treatment* But the risk of rabies only exists after bites which have broken the skin provided the skin has not been broken and there is no bleeding clothing intervening between man and the biting animal removes almost all danger Unless erosions are present or the skin is otherwise broken the contamination of skin by saliva, licking, etc, does not require treatment on the other hand, all contact with the nasal labial, genital or other mucosa must be considered as creating a serious risk

Use of hyperimmune sera

Tests for the prevention of treatment of [illegible] um
immunized animals have bee[illegible]
therapy Innumerable rese[illegible]
However the advantage of [illegible]
when, by the preparation [illegible]
shown to be possible, under [illegible]
against rabies, even a [illegible]
serum containing a [illegible]
before it penetrated into the [illegible]

Great hope is now placed on the use in man of concentrated hyper immune horse serum which to be effective, should be injected as soon as possible (i e , within 24 hours or at the latest within 72 hours of the infecting bite), the classic antirabies treatment being started 24 hours after injection of the serum It is expected that the experiments under way which have not yet been confirmed, will result in lengthening the incubation period of rabies and in putting an end to treatment failures due to rabies infections with an incubation period shorter than 20 days It may also be possible to reduce the number of vaccine injections necessary after the serum injection if they could be reduced to 6 or 7, paralytic accidents might be eliminated

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FIELD CONTROL OF RABIES

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The rabies virus, as far as is known is pathogenic for all warm blooded animals Natural infection has been found in diverse groups of wild and domestic animals, and the relative importance of these animals as spreaders of the disease varies in different localities Certain wild animals such as the fox, jackal wolf skunk, and mongoose are troublesome in certain areas, fruit-eating and insectivorous bats are a special problem in some parts of Latin America Domestic livestock (cattle sheep goats equines), although frequently affected, are not important as spreaders of the disease In almost all countries, however and certainly in Europe by far the most important transmitter of rabies is the dog

Experience has shown that complete eradication of rabies can be accomplished, and a state of freedom from the disease can be maintained, in most instan... dog Examples in Europe are the ... Northern Ireland, Eire and the ... ne measures alone are used Until the 19th century rabies in northern European countries was essentially a rural disease, with the wolf as the chief transmitter The disease became urban when man and his accompanying dog moved to the cities, and in Europe today the disease is primarily a problem associated with the dog This increases considerably the chances of successful eradi cation when we consider certain epidemiological aspects of rabies as it affects this animal

The dog is infective for only a very limited period of time In the furious form of rabies, the virus may not appear at all in the saliva, and when present it is there from only a few days before observable clinical signs to the time of death within one or, less commonly, two weeks later About one half of canine rabies is usually of the paralytic type and this form is not of great significance from the standpoint of transmission By quarantine measures, elimination of stray dogs and now with the aid of effective vaccination, we can encourage the natural trend of this disease

towards self-limitation and assure ourselves that any smouldering sparks of infection will be snuffed out by arithmetical reduction in the possibility of spread

Before considering the mechanism of field control programmes, a brief consideration of symptomatology and vaccination in dogs might be useful A brief reference will be made to vaccination procedures as applied to other animals

Clinical Signs in the Dog

The clinical signs of rabies in dogs can be summarized as follows

Usual incubation period 3 8 weeks, depending on amount and site of inoculum , it may be as short as 10 days, or protracted to one year Clinical signs last 3 7 days, occasionally longer (10 11 days) Change of normal disposition—friendly to unfriendly, or vice versa Alert, troubled look Unfriendly with other dogs and man, although may become unusually friendly with owner Restless, "fly-snapping" Licks or gnaws at bitten part Pupils dilated and may be unequal in diameter, photophobia, decreased corneal reflex Dysphagia, salivation, increased sexual desire Periodic hyperexcitability, hyperaesthesia the animal is fearful, and often hides Bradycardia (pulse rate 40 60 per minute), eye congestion, slight fever Clinical types—furious, or dumb (paralytic)—are recognizable on third to fifth day

Furious form

Animal wanders aimlessly but often in straight lines, blindly bumps into objects Signs of excitability or irritability Restless in enclosed spaces, wishing to escape, snaps or bites at anything, barks continually as if in pain Fine tremors almost always present, often accompanied or followed by spasmodic muscle contractions Perverted appetite—eats cloth, stones, sticks Usually avoids man unless attacked Bark howl followed by hoarse barks of lower pitch Glazed eyes, loss of corneal reflex May stray long distances snapping and biting, may return home and act almost normally for a while

Muscle paralysis, salivation convulsions May lose "voice" entirely Lower jaw hangs fixedly, dirty and discoloured tongue protrudes, drooping head Ataxia, weakness of hindquarters, paralysis, death follows shortly Paralytic manifestations often related to point of entry of virus—head and neck bites cause paralysis of jaw before hind limbs are affected, conversely with bites on hind limbs

Dumb (paralytic) form

Owners often feel that dog "has chicken bone in the throat" Animal rarely bites, not irritable, lethargic hides, muscle tremors Paralysis of lower jaw followed by complete paralysis and death Clinical course 3-4 days—shorter than furious form

Less common signs

"Running fits", animals develop convulsions of short duration with complete but temporary recovery local paralyses salivation, trismus, and haemorrhagic gastro enteritis may rarely be the only signs "Silent" rabies—interval of 10 20 days between first stage and death, dog may eat or drink up to a few hours before death, and paralysis may be absent In peracute rabies dog may die suddenly within 3 days without paralysis or other symptoms, and may even swallow food until death

Vaccination

In 1940, Gautier [1] reviewed the position up to that time of the preventive vaccination of dogs before exposure to rabies He, as well as Webster [7] in 1942, pointed out the unsatisfactory bases upon which the claims of both the supporters and opponents of canine vaccination were formulated One of the major difficulties in the critical evaluation of rabies vaccines was the incomparability of available data The great number of challenging procedures, strains of virus, methods of vaccine production, and kinds of animals used experimentally in addition to the widely varying field conditions under which vaccines were tried had yielded heterogeneous and conflicting results The notable exceptions to the mass of data of doubtful value available at that time, however, were the outstandingly successful mass prophylactic vaccinations in dogs as performed in Japan in the 1920's and in Hungary in the 1930 s

During the past ten years more carefully controlled and statistically significant experiments (particularly those of Johnson, Koprowski, Tierkel, and their co-workers [2, 3 4 6]) have demonstrated the value of single inoculation procedures for canine prophylaxis with both living and killed virus vaccines On a practical level, the efficacy of single-inoculation prophylaxis has been corroborated by the extensive and successful field use of killed vaccines in the United States of America, the continued success with living fixed virus vaccines as used in Hungary and Poland, and the excellent results being obtained in Israel, Malaya, Mexico, Southern Rhodesia, the USA and elsewhere with the living modified virus vaccine, prepared in chick embryos with the Flury strain of rabies virus Tierkel [6] has summarized the results of carefully controlled laboratory experiments

TABLE I RESULTS OF INOCULATION OF DOGS WITH RABIES VACCINES PREPARED BY DIFFERENT METHODS

Preparation of vaccine	Rabies mortality ratio (deaths per total number of dogs)	Period between vaccination and challenge (years)	Workers
Phenolized 20% suspension nerve tissue	6 52	1	Johnson[3]
Controls	41 52		
Chick embryo (Flury)	0 25		
Phenolized 20% suspension nerve tissue	3 22	1	Koprowski & Black[8]
Controls	18 25		
Chick embryo	3 25		
Phenolized 20% suspension nerve tissue	8 19	2	Koprowski & Black[9]
Controls	21 23		
Chick embryo	0 32		
Phenolized 20% suspension nerve tissue	0 30		
Ultra violet light irradiated	0 31	2	Tierkel[4]
Benzene-ether extracted calcium acetate washed	0 30		
Controls	18 33		

in dogs using rabies vaccines prepared by different methods, his data are reproduced in table I, with slight modifications so as to incorporate parts of his text

It is important to point out that all the vaccines cited in these tests were tested for potency before use (Habel mouse test for potency for all but the chick-embryo vaccine, which was tested for potency in guinea pigs, as the mouse is not a suitable test animal for this type of vaccine) This aspect is stressed because rabies vaccines not subjected to adequate tests for potency have given very poor results in the laboratory and in the field, and have often resulted in the past in unjustified rejection of canine rabies vaccines in general

The fact that emerges clearly from the above data is that one inoculation of a vaccine adequately tested for potency gives excellent protection to dogs for at least one to two years As yet no definite statement can be made as to which vaccine gives the best and longest protection, but further experiments now under way might give us some indications within the

next year. Chick-embryo vaccines however have the advantage of cheap production costs, once the technique of production has been mastered.

Unfortunately, similar data are not available with reference to the vaccination of livestock such as cattle horses sheep, and goats. These animals are not usually vaccinated before exposure to rabies. In limited experiments Koprowski (personal communication) has found that three dog-doses of Flury-strain chick embryo vaccine given intramuscularly before exposure will protect cattle against rabies infection. In limited post-exposure experiments in dogs he has shown that hyperimmune serum in combination with vaccine will give good protection. Further work is in progress on post-exposure treatment of dogs and cattle. Although ost exposure inoculation of vaccine alone is commonly used for large vestock in different countries there is no experimental evidence that this procedure is effective.

Field Control

An excerpt from the report on the first session of the WHO Expert Committee on Rabies concerning the control of rabies in an ... is annexed to this paper (see page 233) ... will be given ... In cou ... is for im ... v ... cats are feasible ... there is no question that such quarantine measures measures are the most effective means of keeping a country free of rabies. Quarantine measures are particularly suitable for islands, such as Great Britain, where a six-months isolation and observation period for imported dogs and cats has been employed so successfully.

The field control of rabies in an affected area will not be successful if only half measures are applied. A striking example of this fact is the experience encountered a few years ago in Tennessee (USA) where, despite thorough dog-control measures over a period of years, rabies persisted and became more prevalent until an intensified dog vaccination campaign was introduced.[6] Half measures usually result when there is a lack of co-ordination and liaison between departments of health and of agriculture—the two official groups mainly concerned in the control of rabies. This lack of co-ordination is the compelling reason for the establishment of "a central authority headed by a public-health officer, preferably a veterinarian, who has full executive power and who devotes his full time to this work" (see Annex 1, page 233). It is in such a position that the public-health veterinarian can be of greatest service in mobilizing the resources available to carry out a successful programme. When rabies has been brought under control and a good reporting and diagnostic system has

been established for future contingencies, the veterinarian can be relieved of his full time assignment and can devote *his major efforts* to other projects

The fundamental requirements for bringing an outbreak of rabies under control are

(1) *Registration, licensing, and taxation of dogs*

This procedure can and should be combined with

(2) *Mass vaccination of dogs with a vaccine tested for potency*

The objective should be the vaccination of at least 70% of the dog population within the shortest possible period of time—say, two weeks Mass-vaccination campaigns require the mobilization of all available veterinarians in the district and the setting up of vaccination clinics at convenient locations at specified times Maximal publicity must be given to this part of the programme If at all possible vaccinations should be *free of charge* to encourage the co-operation of the public The fees collected for (1) above can be used to offset the expense of vaccination When mass vaccinations are successfully accomplished, the drop in cases of rabies is often dramatic within three to four weeks

(3) *Elimination of stray animals*

Teams should be assigned especially for this purpose and should work intensively during the registration, licensing, and vaccination period Every effort should be made to ensure the humane destruction of dogs in order to avoid unfavourable repercussions from animal protection societies Carbon monoxide poisoning is perhaps the safest, most convenient, and most humane method After the active campaign is finished, residual units for collection of stray animals should be *maintained* as a permanent activity

(4) *Adequate diagnostic facilities*

Simple instructions should be prepared for health and veterinary officers, describing the preparation and forwarding of suitable specimens to diagnostic laboratories It is preferable to select one diagnostic laboratory to serve relatively large areas, since the laboratory diagnosis of rabies is a specialized procedure It is important, however, to make [illegible] to be con[illegible] located [illegible]

[illegible] veterinarians can be used for this purpose Police stations are also often useful in this connexion

(5) Reporting of the disease

Steps should be taken to make rabies a notifiable disease It is best to incorporate this with the official statistical services of the health and veterinary departments All bites by animals should be reported to the health authorities, as well as all clinically confirmed or laboratory-proved cases of the disease These reports should be disseminated to physicians and veterinarians weekly

The restraint of dogs and cats during the campaign, as stated in Annex 1, should not be forgotten The reduction in number of wildlife species, where these are a reservoir of rabies, is not as applicable to European countries as it is to other areas of the world The control of wildlife species is a difficult task, and much remains to be learned about the procedures involved Trapping, shooting and poisoning are the common methods of attack, but these measures must be adapted to local conditions

It is safe to say that the success or failure of a rabies-control campaign will depend on the quantity and quality of publicity given to it Every means should be used to explain in simple terms what is being done, and to encourage the vaccination of dogs Newspapers, broadcasting services, churches, schools, local clubs, and professional and lay societies should be enlisted for this task The population must become and remain "rabies conscious" for the few weeks necessary to bring the outbreak under control A concentration of effort for a relatively short period will pay handsome dividends in practically eliminating a problem which will otherwise drag on interminably

Annex 1

CONTROL OF RABIES IN ANIMALS *

It is recognized that rabies exists in two epizootiological forms (1) a widely disseminated disease propagated principally in dogs predominantly in urban regions, and (2) a more localized disease of wild animals particularly in wolves foxes jackals, vampire bats, and mongoose

The application of known effective measures for the elimination of rabies from the dog population constitutes the most challenging problem at this time in that this animal is the principal source of human infection

The committee has considered the answers to the questionnaire concerning the control of rabies in animals and notes the general agreement on the main principles to be applied in rabies control On the other hand there are wide differences of opinion expressed with regard to fundamental details such as the length of quarantine periods,

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* Excerpt from the report on the first session of the WHO Expert Committee on Rabies World Hlth Org techn Rep Ser 1950 28 11)

and the testing and application of veterinary vaccines The committee wishes to record therefore their recommendations on these various problems *with the realization that* the application of the various measures proposed will have to be adapted to local conditions

The committee recognizes the distinct value of periodical compulsory prophylactic vaccination of dogs against rab es and recommends its use in areas in which the disease is enzootic

During the past ten years carefully controlled experiments have demonstrated the value of a single dose of either living or inactivated virus vaccine repeated annually From a practical point of view the efficacy of this procedure has been corroborated by the extensive and successful field use of inactivated virus vaccines in the USA and the *continued success with living attenuated virus vaccines in Hungary*

The committee recommends that vaccines be subjected to adequate tests for potency It is of the opinion that the presence of live virus in vaccines is not of itself sufficient

different problem in potency testing which has now been adequately met This vaccine has proved to be highly antigen c in tests in guinea pigs and dogs

The committee recommends that where feas ble a biting animal should be kept under observation for a period of ten days If the animal shows no signs of illness during this period it can *safely be assumed that the animal was non infective at the* time of biting

The committee further recommends that during an outbreak of rabies if general restrictive measures alone are depended upon in an involved area dogs should be restrained (leashing secure confinement) for a minimum period of 90 days from the date of the last known case of rabies Where in addition to restrictive measures vaccination of dogs is carried out the period of restraint may be reduced to 30 days after vaccination The restraint of domestic cats is not feasible

The committee recommends that dogs and cats bitten by a rabid animal should be destroyed If the owner is not willing to destroy the exposed animal the following alternatives are recommended

(*a*) strict isolation of the animal in a kennel for a period of 6 months

(*b*) if no previous vaccination has been given within a period of 12 months vaccination and confinement in a kennel for 3 months

(*c*) if the animal *has been previously* vaccinated within 12 months revaccination and restraint (leashing secure confinement) for 30 days

milk from infected animals Because [illegible] does not feel it possible to make any specific recommendation with respect to the vaccination of large animals after exposure

The committee recognizes that countries now *free of rabies should continue either* [illegible] ıbject them to a prolonged period [illegible] entry In the case of countries [illegible] eady present in domestic or wild [illegible] easures are impracticable There

can be no objections to the importation of dogs from countries free of rabies, provided they have been isolated en route Dogs originating in infected countries should be vaccinated within 12 months before departure, and revaccinated as soon as possible after arrival, by whatever procedure is practical in a particular area

These recommendations are made in consideration of the varied conditions encountered throughout the world, and should not be construed as discouraging more stringent measures, such as quarantine periods upon entry, with (preferably) or without vaccination

Experience has shown that the efficient organization of a rabies-control programme in an infected area is best accomplished by means of a central authority headed by a public-health officer, preferably a veterinarian, who has full executive power and who devotes his full time to this work A system of weekly reports of rabies cases should be instituted to enable the officer to keep abreast of the problem He should enlist the support of all local groups directly or indirectly concerned with rabies, such as public-health authorities, veterinary and medical practitioners, livestock organizations, animal protection societies, etc These groups can provide material assistance to the rabies-control officer by publicizing the programme and otherwise informing the general public whose co-operation must be obtained before specific measures can be successfully applied If possible, an antirabies campaign should be co-ordinated on a national basis, or at least in adjacent infected areas

The committee recommends that the following specific measures be applied in affected regions

(1) Registration, licensing, and taxation of dogs
(2) Elimination of stray animals
(3) Restraint of dogs while the control campaign is under way
(4) Mass vaccination of dogs free of charge
(5) Provision of adequate facilities for diagnosis
(6) Reduction in number of wildlife species where these are a reservoir of the disease
(7) A continual and energetic publicity campaign

Annex 2

CONTROL OF RABIES IN FOXES IN NEW YORK STATE, USA *

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In the early 1940's, it was felt that the wildlife phase of the rabies problem was incidental to the disease in the dog This belief was strengthened by what happened in the western part of New York State in 1943, 1944, and 1945. Here the disease among foxes disappeared spontaneously while among dogs it continued to spread over a considerable area

* This paper was prepared by Dr Zeissig in March 1950, for the first session of the WHO Expert Committee on Rabies, and is reproduced here because of the difficulties encountered with fox rabies in some European countries

In the central part of the State, what appeared initially to be a similar situation began to develop about a year later However, the disease continued to spread among wild animals Extensive countywide vaccination programmes conducted in this area have almost eliminated rabies from the dog population Indeed, it can safely be said that, were it not for a continuation of the disease among wild animals, the problem in dogs would have been solved at least two years ago

After having established that the wildlife reservoir was the key to the problem in this area, a method was evolved during the succeeding years which appears to be effective in controlling this disease among wild animals In 1946 the plan was to surround the *infected area with a line of traps for the purpose of removing* as many foxes as possible from a belt around the infected area The hope was that this zone would be sufficiently deep for a rabid fox crossing it from the inside to succumb without having had an opportunity to transmit the disease to others of its kind This method appeared to function for a time It was tried only on a small scale, but the disease circumvented the end of the trap line Essentially the same procedure was then tried on a larger

scarcity deeper This scheme appears to have been successful, but before passing that judgement one should wait at least a year before regarding an area as free of infection

In the meantime, however, there has been a spread of the disease from the general area back to localities which have been free of the disease for about a year In some instances this constitutes a *reversal of direction of spread This is believed to be due to the restora*tion of the fox population in areas once depleted by the disease It has

period of the disease, wer

for seven weeks confine

trapping in townships in which rabies exists to

foxes If this legislation becomes *law, it will allow both "strategical trapping"* in areas bordering infected territory and "tactical trapping" within already infected areas

It is felt that this reduction in the fox population operates in a fashion similar to the vaccination of dogs, where the establishment of large numbers of immune dogs

successfully prevents the spread of the disease If the reduction in the number of foxes is sufficiently great, e g 20% 30% of a normal population the survivors will usually be sufficiently scattered for the disease not to be readily transmitted among them

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METHODS FOR THE DIAGNOSIS OF RABIES

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In principle, the diagnosis of rabies consists in recognition of the presence in an animal of acute encephalomyelitis which can be ascribed to the specific agency of the rabies virus

In practice, a distinction must be made between whether a person has been bitten, and may consequently need treatment, or not, for on this distinction depends the urgency of the diagnosis

Procedure in Cases of Bites

If a person has been bitten, diagnosis is urgent, since the course to be followed depends upon it, and the results of inoculations cannot be awaited Various possibilities have to be considered

1 *Animal is alive and apparently healthy*

The animal should be kept under observation and should on no account be killed If it is suffering from rabies, the symptoms usually appear within 48 hours and almost always within a week (maximum period observed 14 days) Except in cases of bites on the face, treatment may be postponed and the diagnosis deferred

As soon as the initial symptoms of rabies appear, the second possibility has occurred

2. *Animal is ill*

It is then possible to make a clinical diagnosis Usually there are characteristic symptoms paresis of the hindquarters or of the jaw in cases of paralytic rabies, fits of aggressive dementia, salivation, and hoarseness of the " voice " in cases of furious rabies Treatment of bitten persons should be started immediately without awaiting confirmation of the diagnosis Usually the animal dies in two to three days If the death

middle layer of the neuroganglia of the Ammon's horn, and, less frequently, in the neurons of the cerebellum, of the motor areas, and of the medullar nuclei They may be present in very large numbers in the ganglia but are generally small in size

The lesions of fixed-virus rabies are found exclusively in the middle zone of the outer layer of the cells of the Ammon's horn They always co-exist with lesions of street rabies but in a variable proportion

These lesions can only be detected by special staining-methods, such as the Mann, Giemsa, or Sellers methods Their presence enables a definite positive diagnosis to be made

Procedure in Cases with No Bitten Persons

This procedure is also applicable in the case of bitten persons already receiving treatment

Diagnosis is not a matter of urgency it consists of identifying the virus There may be no clinical evidence for a diagnosis of rabies The virus to be identified may be one isolated by chance from an animal or may be the subject of postmortem examination in the case of a person who has died of encephalomyelitis, or, again, it may have been encountered during epizootic surveys, etc Diagnosis is then based upon

1 *Isolation of virus*

This is done by inoculation of a laboratory animal Depending on what technical facilities are available the animal should be a rabbit, guinea pig, hamster, or mouse The last is the most economical having the shortest incubation period However, symptomatology in the mouse may be atypical, and if the doses of virus inoculated are too weak the animal may survive beyond the normal period, although showing characteristic histological lesions

The inoculation material should preferably be the brain of the suspected animal However, with the dog the salivary gland may also be used care being taken to select the submaxillary gland which is the most accessible and the most consistently virulent

The inoculation material should be prepared and inoculated as described in Annex 4, page 254

The inoculated animals should be placed in isolated cages and kept under observation for 40 days and in no case for less than 30 days, street rabies usually breaks out in inoculated animals between the 12th and the 25th day after inoculation, and in the mouse sometimes as early as the 5th or 6th day

If the animals fall ill or die, the following investigations are carried out

2 *Search for specific lesions of the central nervous system*

This is carried out as indicated above

3 *Identification of the virus isolated*

This identification can be made directly by neutralization of the unknown virus with a known rabies antiserum whose titre has been checked (see Annex 5, page 258) provided that assessment of the result is based on a correct technique and on significant differences (see Annex 6, page 264) This test is necessary and sufficient

The virus may be identified indirectly by vaccinating animals, particularly rabbits, with the attenuated unknown virus (phenolized vaccine) and seeing whether injection of that vaccine brings about the appearance in the animal of antibodies which neutralize or fix a known rabies virus of tested titre (fixed virus) In this case the investigation amounts to carrying out the titration of an antirabies serum This is usually done by inoculating an animal, such as a mouse, with virulent emulsions of decreasing concentration, neutralized with fixed quantities of the serum under test, and comparing with a normal serum (see Annex 5, note 4) The complement fixation method may also be used if a rabies antigen specially prepared for the purpose is available This method is not in routine use, however, and for the time being is within the scope of specialized laboratories only

Annex 1

BRAIN REMOVAL AND PREPARATION OF MATERIAL FOR EXAMINATION FOR NEGRI BODIES (IMPRESSIONS, SMEARS, AND SECTIONS)

Large Animals (Dogs, Cattle)

Sacrifice the animal by gassing with coal gas or by bleeding Avoid the use of anaesthetics, such as ether, which cause artificial congestion of the cerebral capillaries, and of poisons, for instance, strychnine, which interfere with subsequent animal inoculation If the head has to be sent some distance, it should be kept cold by placing it in sawdust in a tightly

closed container, placed in a box packed with cracked ice. Avoid freezing (with dry ice, for instance), which delays and may interfere with histological examination. The head should be sent immediately to the laboratory concerned, accompanied by detailed information on the conditions under which it was removed, the symptoms shown by the animal, the period of observation of the animal before death, whether the animal died or was killed, whether persons were bitten, etc

Removal of brain

Equipment

- 1 pair of bone-holding forceps
- 1 pair of bone-cutting forceps
- 1 saw
- 2 pairs of serrated dissection forceps
- 2 pairs of large scissors
- 2 pairs of medium sized scissors
- 2 pairs of flat jawed forceps
- 1 brain-knife
- 1 scalpel
- 2 large Petri dishes

The animal is firmly secured to the autopsy table, or better, the head is separated from the body and strapped to a wooden block hollowed out for the purpose The operator and his assistants should wear thick rubber gloves to protect their hands Make an incision on the mid-line of the skull through the skin, fold back the skin flaps, and free the muscles and fascia as far as the base of the skull, proceeding from the crown to a horizontal line passing through the eyes Saw transversely through the skull at eye level and symmetrically through the occipital bone, saw through the temporal bone on either side of the skull level with the cuts already made Link the saw cuts with the bone-cutting forceps, lift the skull-cap, and push it backwards In the case of very large animals (large dogs, cows, etc) a different method is preferable make a longitudinal saw-cut on each side of the mid line and at about 1 5 cm from it, and link these cuts by one or two transverse saw-cuts above the orbits and at the occiput so that the crown can be removed in two symmetrical pieces

Once the crown has been removed, take fresh instruments and open the meninges, using serrated dissection-forceps and a pair of fine, sterile scissors The operation is performed by making an incision in the meninges, starting from the middle, along and on each side of the longitudinal sinus, a second incision is made perpendicular to the first and the meningeal flaps are folded to the front and rear The instruments are changed again Cut through the medulla as low as possible with a scalpel and lift the brain

forwards, successively severing the pairs of cranial nerves At the end of the operation the brain is tipped forwards into a large sterile Petri dish where it rests on its upper surface In hot weather or if the brain is soft, as in the case of an animal which has not been sacrificed but has died from natural causes, it should be cooled to + 5°C in a refrigerator to give it a firmer consistency before dissection

Examination of brain

Note whether there is congestion of the cerebral vessels or exudate in the meninges etc Dissect the brain as follows

(1) With a brain knife separate the two hemispheres longitudinally, after detaching the cerebellum and medulla

(2) Find the hippocampus and the Ammon's horn This may be done in one of the following two ways (*a*) Cut across the brain transversely, starting from the base behind the optic chiasm and proceeding towards the lower third of the convexity, the third ventricle appears on the cut surface and the Ammon's horn is seen as a whitish fold resembling a large bean cut transversely, and can easily be removed (*b*) Make a longitudinal incision externally in the posterior third of each cerebral hemisphere about 1 5 cm from the mid-line, the incision is continued through the grey matter and the white matter until the third ventricle is reached in the form of a narrow fissure The hippocampus will be seen on the base of the ventricle in the form of a glistening, white, semi-cylindrical bulge, extending laterally on each side

Cut transverse sections 1-2 mm in thickness from each hippocampus Take similar samples from the cerebral cortex (motor area), the cerebellum, and the medulla

When impressions are to be examined, at least six slides (two for each hippocampus, one for the cerebral cortex, and one for the cerebellum) should be carefully inspected before deciding that the results are negative If the results are negative, however, a histological examination is carried out

Preparation of impressions

Place a fragment of brain (cortex, hippocampus, cerebellum, etc) on a piece of filter paper in a Petri dish and cut the upper surface of the fragment with a scalpel so as to give it a surface area of about 1 cm × 0 5 cm A very clean histological slide is then applied to the cut surface, using just sufficient pressure to spread the section out slightly on the slide so as to leave an impression of the cut surface Make three or four impressions on the same slide While the preparation is still moist, stain it by Sellers's method (see Annex 2, page 248)

"Spread smear" preparation

With this method, which is not recommended, a small fragment of the tissue to be examined is removed, crushed between two microscope slides, and spread out in the same way as a blood smear. The method usually produces smears which are too thick and completely lacerates the tissue, resulting in the expulsion of the Negri bodies from the neurons and in making them difficult to identify

A variant of the above methods, which may be employed with good results, consists in placing the slice of tissue on the small end of a new cork—or on a wooden spatula, such as a tongue depressor—and spreading out the peripheral tissue with a match stick over the surface of the cork, thus increasing the degree of adherence and making the grey matter stand out Taking the cork between the thumb and index finger, make impressions on a slide as indicated above Next, using a clean match-stick remove a fragment of grey matter from the centres of the sample and gently roll the match stick on one or more clean slides In this way, the same fragment can be made to yield impressions and smears, the latter being generally thinner than those obtained by the usual method

Preparation of tissue samples for histological examination

At the same time as the impressions and smears are made, material which it may be necessary to keep for histological examination is removed From the areas exposed as described above are removed thin slices of tissue (about 2 mm thick), with a surface area corresponding to that of histological sections, which are immediately submerged in the fixing agent selected (see Annex 3, page 249) If the tissue is soft and difficult to section, prepare pieces of filter-paper slightly larger than the cut brain sample to be collected Apply the piece of filter paper to the cut brain surface, with the left hand hold the edge of the filter-paper with fine forceps, and with a scalpel held in the right hand cut out a piece of brain parallel to the filter-paper and 2 or 3 mm from it, the section of brain is immediately submerged in the fixing agent together with the fragment of filter-paper to which it adheres

Removal of material for inoculation

In operating, care should be taken to put on one side fragments aseptically removed from the same areas (cortex, hippocampus, cerebellum, medulla) for use in animal inoculation (see Annex 4, page 254). If the brain is received in good condition and can be assumed to be sterile, the fragments should be removed before any examination is made When the brain is infected, the fragments may be removed at any time and antibiotics added, as described later

Small Animals (Rabbit, Guinea-pig, Hamster, Mouse)

Rabbit

Equipment

- 1 pair of Liston's or Pasteur's forceps
- 1 pair of Farabeuf's forceps
- 1 pair of serrated dissection forceps
- 2 pairs of large scissors with pointed blades
- 1 scalpel
- 1 pair of fine dissection forceps
- 1 pair of fine scissors

The animal, resting on its ventral surface, is attached to the autopsy board, with the head at the edge of the board Using the serrated dissection forceps, the scalpel, and the scissors, completely scalp the head from the nape to the muzzle, removing the ears and the upper eyelids Moisten the exposed surface of the head with iodized alcohol and rapidly flame it with a bunsen burner Holding the muzzle of the animal with the Farabeuf's forceps in the left hand open the brain pan with three cuts of the bone forceps Make the first two in the front part of the head, from each orbit to the mid line with an upward and outward movement, opening the brain pan (parietal and temporal bones) in two flaps, to the right and to the left The third cut is made at the occiput, with a backward eversion movement, which completes the clearance of the field The Farabeuf's and bone forceps are then laid aside With the fine forceps and the scissors, the meninges are cleared aside and the anterior part of the brain sectioned at the olfactory lobe, the medulla is severed level with the cerebellum, and the brain having been raised in order to cut the *optic chiasm*, it is placed in a Petri dish where it is dissected

With the brain lying on its dorsal surface in the Petri dish, the ventral surface facing upwards, cut the brain stem at the peduncle, then cut through the encephalic mass obliquely along a transverse slanting plane, starting from the optic chiasm and going towards the convexity, parallel to the posterior surface of the hemispheres and to the cut surface of the brain stem A second cut made in the same way, parallel to the first and 5 8 mm behind it, gives a transverse section of the brain which includes the gyrus hippocampi and the Ammon's horn as well as the basal optic ganglion, areas of choice for the detection of Negri bodies, in addition to the cortical motor area A transverse section of the cerebellum makes it possible to examine Purkinje's cells and the peduncular region Finally, a slice cut from the end of the brain stem gives a section of the medulla To reach the gasserian ganglion, cut through the petrosa with the bone forceps where it connects with the sella turcica, make the cut surface

freed of bone fragments, placed on a piece of filter-paper, and immersed in the fixing agent with the other samples

Guinea pig and hamster

Equipment

2 pairs of large scissors
1 pair of serrated dissection forceps
1 pair of fine forceps
1 pair of fine scissors

The animal is secured to the autopsy board and the head thoroughly freed of skin The skull is rapidly flamed With the second pair of large sterile scissors, open the skull by means of four incisions round the brain pan, the first one linking the two orbits and the other three on the sides and the occipital bone Remove the brain with the fine forceps and scissors and dissect it as described for the rabbit It is more difficult to locate the gasserian ganglion in the guinea pig or hamster than in the rabbit, but with care it can be done successfully

Mouse

Equipment

1 pair of serrated dissection forceps
1 pair of medium scissors
1 pair of fine forceps
1 pair of fine scissors

The mouse is pinned down by its ventral surface on a sheet of cork, the limbs being spread out and stretched First, the four paws are stretched and pinned, the base of the tail is then secured with a fifth pin and a sixth is passed through the anterior extremity of the muzzle which is firmly stretched forwards Remove the skin well back from the head Treat the skull with iodized alcohol and flame it very gently with the pilot flame of the bunsen burner Open the skull with the fine scissors, starting by linking the orbits and then cutting laterally through the skull fairly low and finally pushing back the flap thus obtained Sever the medulla and the chiasm Remove the whole of the brain mass and place it in a Petri dish Separate the cerebellum Cut through the brain traversely at the optic chiasm and along a plane parallel to the posterior surface of the brain, so as to obtain a cross section which is placed in the fixing agent, the anterior portion being reserved for smears and inoculations Fix also a section of the cerebellum

If a ganglion is to be examined, the upper part of the spinal cord should be detached and removed together with the upper cervical ganglion

Smears or impressions are made in all cases in the same way as described for large animals

Annex 2

STAINING OF SMEARS FOR DETECTION OF NEGRI BODIES BY SELLERS'S METHOD *

Preparation of Stain

(*a*) *Stock solution of methylene blue*

methylene blue, bacteriological quality	2 g
methyl alcohol (absolute, acetone free)	100 ml

(*b*) *Stock solution of basic fuchsin*

basic fuchsin, bacteriological quality	4 g
methyl alcohol (absolute, acetone free)	100 ml

Keep both the stock solutions and the dilute solutions in the refrigerator

The following dilute solution is prepared as a working stain

methyl alcohol (absolute, acetone free)	25 ml
methylene blue, stock solution	15 ml
basic fuchsin, stock solution	2-4 ml

The methyl alcohol and the methylene blue are mixed in a 50-ml drop-bottle, 2 ml of the basic fuchsin stock solution are added, and a trial stain is made using slides with a smear of brain tissue inoculated with the street virus, fixed in methyl alcohol, and kept dry Macroscopically, a properly stained smear, when held up to the light, should generally appear violet in the thinner areas, shading into purplish blue in the thicker portions

If in the trial stain with the dilute solution the thinner parts are bluish, add 0 5 ml of fuchsin to the dilute solution and make another trial It is rarely necessary to use a total of more than 3 ml of fuchsin solution for the quantities given The staining properties of the mixture improve

* Sellers T F (1927) *Amer J Publ Hlth* 17 1080 The method described by Sellers has been slightly modified by E S Tierkel

after standing for 24 hours and keep indefinitely if protected from evaporation, which tends to make the fuchsin become too dominant Cold storage helps to maintain the stability of the solution If the solution becomes concentrated by evaporation the addition of absolute methyl alcohol will restore the proper balance to the two dyes Larger quantities of stain than those indicated above may be prepared and may be kept indefinitely in tightly corked bottles

Staining Technique

Prepare the smears or impressions of brain tissue in the way indicated No preliminary fixation is necessary

While the smear is still moist cover it with stain poured from the drop-bottle and allow staining to proceed for 4-5 seconds

Rinse in tap-water and allow to dry without blotting Drying of the stained slide may be accelerated by waving it in the air or by passing it through a current of hot air Carry out microscopic examination first with low magnification and locate the thin areas of the smear showing large nerve cells then examine these areas with the oil immersion lens The Negri bodies which appear purplish red or magenta stand out in bold relief Basophilic structures inside the Negri bodies are usually clearly visible The cytoplasm of the nerve cells is purplish blue, the nuclei and nucleoli dark blue, the stroma pale pink and the nerve fibres a deeper pink the neural sheaths do not stain and the erythrocytes are brick red If present, bacteria from a secondary infection are stained an intense blue

Annex 3

HISTOLOGICAL METHOD FOR EXAMINATION OF SECTIONS FOR NEGRI BODIES

Rapid Corrosive-Sublimate Fixative [4]

We recommend the following for rapid fixation of the nervous system

Mix equal volumes of glacial acetic acid, acetone, and a saturated solution of corrosive sublimate ($HgCl_2$) in absolute alcohol The mixture is made in advance and keeps perfectly Prepare the saturated solution of corrosive sublimate in absolute alcohol in hermetically sealed bottles

hastening saturation by keeping in the incubator at 37°C. The sections should of course be treated with Lugol's solution or iodized alcohol before staining, in order to remove the sublimate. Mixtures containing other components, particularly formol, give distinctly inferior results, as do mixtures into which water or hydrated alcohol has been introduced in order to retard fixation. Tissues other than those of the nervous system are hardened by this fixing agent.

Rapid Method for Histological Embedding

For slices of brain tissue 1 mm in thickness, fixation is complete in 15 to 30 minutes. The tissue is transferred directly to absolute alcohol with which it is treated for 20 to 30 minutes in two baths, followed by two baths of toluene and two of paraffin each lasting 15 minutes. Including the time necessary for cutting and staining the sections, the preparation is thus ready for microscope examination 3½ to 4 hours after the autopsy of the animal.

Embedding with Dioxane

It is sometimes advantageous to use a solvent for paraffin which is also miscible with water. The number of operations is reduced, and for fairly thin tissue fragments there is a gain of time. The dioxane technique is suitable for this purpose.

Fix the tissue in thin slices, not exceeding 5 mm in thickness. In principle, any *fixing agent can be used*. It is *preferable* however, to employ Bouin's dioxane mixture as given by Lison.[8]

dioxane saturated with picric acid	8.5 volumes
commercial formol	1 volume
glacial acetic acid	0.5 volume

Next embed without washing, as follows:[9]

dioxane I	1 hour
dioxane II	1 hour
dioxane III	2 hours

followed by

paraffin I	15 minutes
paraffin II	45 minutes
paraffin III	2 hours

Use fresh paraffin for embedding.

Take care to keep the dioxane in well stoppered bottles, since there is a risk of chronic poisoning, accompanied by anaemia caused by the vapour if the dioxane is kept in open bottles and ensure that it remains anhydrous by adding granules of CaO

Staining of Negri Bodies by Mann's Method

This classic method gives sections which are permanently stained, with very fine differentiation of the Negri bodies It is an excellent demonstration method, but it requires time and a certain knack for full success

Procedure

Prepare the mixture at the time of use

methyl blue (not methylene blue) 1% aqueous solution	18 ml
1% aqueous eosin solution	23 ml
distilled water	49 ml

Stain for 24 hours at laboratory temperature or for 6 to 14 hours at 38°C, in the latter case, first treat the section with alcohol formol to render the gelatin insoluble, since otherwise the sections come loose

Wash first with tap water and next rapidly with absolute alcohol

Differentiate in alcoholic caustic soda solution

1 5% solution of caustic soda in alcohol	1 ml
absolute alcohol	30 ml

As soon as the section is stained pink (about 10 minutes), wash the preparation well with tap water The section should take on a sky blue colour, if not, treat it with water containing acetic acid (2 drops of acetic acid in 40 ml of distilled water) for 1 minute

Dehydrate rapidly in absolute alcohol wash in xylol, and mount in balsam

Result

Negri bodies, vermilion red, nucleoli of the neurons, violet red, chromatin, blue, cells, dark blue, stroma, pale blue, erythrocytes, pink

By substituting phloxin B for eosin in the same proportion, the preparations obtained are less attractive (purplish blue or mauve background instead of sky blue) but the inclusions (Negri bodies) are more numerous and more striking

Staining of Negri Bodies by the Fuchsin-Safranin-Blue Method [3]

Procedure

After fixing well, the tissue is embedded in paraffin, cut into thin sections, and freed from the paraffin Stain for 10 minutes with the following mixture

(*a*)	basic fuchsin	1 g
	50% alcohol	200 *ml*
(*b*)	0 2% aqueous solution of safranin	

Mix equal parts of (*a*) and (*b*) *in a drop-bottle the mixture is fairly* stable and keeps for some time

Discard the stain, cover the section with equal parts of alcohol and acetone to remove excess stain, and wash rapidly the section is coloured red Stain for 15 seconds to 1 minute with a 10% dilution of Unna's polychrome blue or with permanganate blue prepared by Stevenel's method [11] used undiluted Discard the stain the section is deep violet in colour Differentiate in alcohol acetone for a few seconds the section *becomes blue, immediately wash the preparation in running tap-water* to remove the excess blue stain and again treat with alcohol acetone, next, without washing, commence dehydration by shaking in a Borrel tube filled with absolute alcohol The remaining stain is removed from the section which becomes differentiated, taking on a lilac pink tint varying in paleness according to the thickness of the section Rapidly complete dehydration in absolute alcohol, carefully remove the alcohol in several changes of xylol and mount in balsam

Result

Stroma very pale pink with nerve fibres a deeper pink neuroganglia and leucocytes purplish blue neurons, light blue, chromatin, deep purple with the nucleolus a vivid red, pathological formations particularly well shown nuclear inclusions and oxyphilic substances, vivid pink, Negri bodies, poppy red to mauve pink, with the internal structure lilac

Detection of Negri Bodies with the Fluorescence Microscope

This method makes use of the fluorescence of the Negri bodies when they have been impregnated with a fluorochrome and the latter is excited by light with a wave length of 4,000 Å [5] When equipment for fluorescence microscopy is available, this is a rapid *and sure method of rabies diagnosis*

1 Fixation of the brain tissue and embedding in paraffin as usual Fine sections are made which are mounted on slides and treated successively with xylol absolute alcohol and water to remove the paraffin

2 Stain by immersion for 30 minutes in a 0 2% aqueous solution of thioflavine S Without washing it treat the preparation for a few seconds with absolute alcohol and then with toluene Mount in balsam between the slide and the cover slip applying as thin a layer of balsam or glycerol as possible It should first be ascertained that the balsam does not contain substances fluorescing in ultra violet light

3 Examine with the fluorescence microscope using a filter to block wave lengths longer than λ — 5 150 Å Locate the Ammon s horn under low magnification and examine with high magnification using glycerol or liquid paraffin as immersion liquid

The Negri bodies immediately stand out because of their vivid fluorescence and brilliant azure blue colour The background of the preparation is pale yellow cellular protoplasm brilliant yellow and nucleoli and erythrocytes pale blue

Other Staining Methods

Among the innumerable methods which have been recommended for staining Negri bodies the following also give good results

1 Lentz s method [2] This is a variation of Mann s method

2 Gallego s method [1] This is a method using acid ferric chloride as mordant followed by staining by Ziehl s method and with picrocarmine

3 Romanowsky Giemsa method This is a buffered Giemsa following the technique of Lillie & Pasternack [7]

4 Stovall & Black s method [12] This method has been modified by Lill e [6]

5 Schleifstein s method [10] This is a combination of Sellers s method with the rapid dioxane embedding method

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hand, by plucking, from the site of injection, and the skin is treated with iodized alcohol

With the rabbit and guinea pig, a 1-ml syringe is used, with a needle 0 7 mm or 0 8 mm in diameter and 20 mm long, for the rabbit, and 0 6 mm in diameter and 15 mm long for the guinea-pig The tightness of the joint between the syringe and needle is ensured by applying a little melted paraffin The needle should penetrate 4 6 mm into the brain

Inoculation of Hamster

The procedure is the same as for the guinea-pig, but the thinness of the skull makes it possible to perforate it directly with a needle 0 6 mm in diameter and 15 mm long

Inoculation of Mouse

For the mouse, narrow 0 25-ml syringes of the " tuberculin syringe " type are used The needle should be 0 4 mm or 0 5 mm in diameter and 15 mm long

The mice are anaesthetized with ether To do this, take a glass jar fitted with a solid cover In the bottom of the jar arrange a platform consisting of a piece of wire mesh turned down at the corners so as to form a horizontal partition a few centimetres from the bottom of the jar Under the wire mesh place a large wad of cotton wool soaked in ether The mouse to be anaesthetized is placed in the jar on the wire mesh, it breathes the ether vapour without coming into contact with the cotton-wool As soon as the mouse is etherized, take it out by the tail, which is held between the fourth and fifth fingers of the left hand, bent into the palm Press the left thumb and index finger on the occipital protruberances behind the ears Rapidly moisten the fur on the head with a pad soaked in alcohol or iodized alcohol, hold the syringe with the thumb, index finger, and middle finger of the right hand, and introduce the needle laterally into the brain half-way between the eye and the ear to a depth of 3 mm Gently inject 0 03 ml Immediately place the mouse in its final cage or jar Inject at least 4 mice Keep the inoculation material until the mice revive and behave normally, showing that they have survived the injury done If they do not survive, make up the number of inoculated mice [1]

[1] Well trained technicians may omit anaesthesia The mouse is held by the tail as described above and allowed to grip a wire mesh jar top placed on the operating table while the operator immobilizes *the head of* the animal with his left thumb and index finger The use of antiseptic to moisten the fur of the head can also be omitted

Sterilization

All equipment which has been in contact with rabies virus should be immediately sterilized Rinse the syringes by drawing up and discharging normal saline several times *keeping the needle under the surface of the* liquid in a container which should itself be sterilized Boil instruments for 10 minutes or after rinsing, place in an enamel pot which is sent for autoclaving

Incubation Period and Symptoms of Rabies in Animals

Inoculated animals should be examined daily and careful note made of the appearance of the first symptoms and of the date of paralysis

Mouse

The incubation period of rabies in mice is usually 8 to 14 days It may be as short as 5 days and rarely exceeds 20 days The first symptoms are staring fur, sometimes the formation of a swelling on the spinal column, and frequently a fairly pronounced degree of excitation, or, on the contrary, an apathy contrasting with the normal behaviour of the animal when an attempt is made to take it in the forceps or the jar is knocked Definite rabies is shown by trembling the appearance of paralysis first in the hind legs and later becoming generalized, and then prostration, soon followed by death It should be noted that with certain strains death occurs rather suddenly and without warning symptoms Since the brain of the mouse autolyses very rapidly after death, the behaviour of the animals should be checked several times daily when the end of the incubation period is reached or when the first deaths in the group have occurred

Hamster

The symptoms in the hamster are similar to those in the mouse, but the paralytic period is generally longer and more pronounced The incubation period of rabies in this animal is hardly longer than in the mouse

Rabbit

The first symptoms in the rabbit are a pupillary dilation preceding by a few hours the respiratory disturbances and the initial paralysis The incubation period, which varies considerably with the different strains of street virus, is normally from 12 days to 25 days As typical lesions are established in the rabbit as soon as paralysis appears the animal should

be sacrificed when it is tetraplegic and prostrate to ensure sterility of the brain, which may be invaded by secondary infections if the agonal period is prolonged

Guinea pig

The incubation period in the guinea-pig is shorter than in the rabbit, and when the first symptoms of rabies appear a period of excitation is often observed during which the animal may become aggressive, and there is some risk of being bitten The animal can be sacrificed as soon as paralysis becomes generalized

Note Animals which have been used for research on the rabies virus and have survived should in all cases be sacrificed at the end of the observation period (40 days), under no circumstances should they be used for other research, not even on rabies

Annex 5

IDENTIFICATION OF RABIES VIRUS BY THE NEUTRALIZATION TEST

This test consists in ascertaining, by means of the neutralizing action of antirabies serum, whether rabies virus is present in virulent material, in order either to identify an unknown virus (rabies diagnosis) or to make sure that a strain of rabies virus maintained in the laboratory has not been replaced by a contaminating virus (lymphocytic choriomeningitis, mouse encephalomyelitis, infectious ectromelia)

Equipment

1 pestle and mortar
2 Petri dishes
1 pair of fine forceps
1 100-ml Erlenmeyer flask
1 bottle of sterile normal saline
1 10 ml pipette with 0 1 ml graduations
3 5-ml pipettes with 0 1-ml graduations
10 2 ml pipettes with 0 05-ml graduations (or 10 1-ml pipettes)
sterile test tubes
inactivated normal horse or guinea pig serum
inactivated antirabies serum
control serum from the same animal species

Mice should be used and should be albinos coming from a stock free from spontaneous infections, they should be in good health Since all strains of mice are sensitive to the rabies virus, it is not necessary to use any special strain, however, all the mice used for any one test should belong to the same strain so as to eliminate individual differences It is preferable to use mice 3 5 weeks old weighing 8 15 g at the time of inoculation Either males or females may be used

Inoculation Material

This is usually taken from the brain of an animal which has died from an infection considered to be rabies Nevertheless the salivary glands may also be used, care being taken to select the submaxillary glands

Sera

All the sera employed, whether specific or controls are inactivated in a water bath at 56°C for 30 minutes The specific sera and the normal sera used as controls are usually kept frozen in sealed tubes They may also be kept at a temperature of 4°C in tubes fitted with a rubber or cork stopper In this case, 0 01% merthiolate should be added to the serum It is convenient to use bottles with perforatable rubber stoppers of the penicillin bottle type so that the serum can be drawn up without opening the bottles It is essential that the control sera (non neutralizing) come from the same kind of animal as the specific serum and have been kept under the same conditions of bottle stopper and temperature, and where applicable, of addition of antiseptics (merthiolate) Cotton plugs should be avoided since they may lead to important errors through evaporation of the serum absorption of vapours or substances which may have a viricidal effect, or variation in pH with changes in the CO_2 content

Preparation of Virus Suspension

The infective material is prepared in the form of a 20% suspension It is first weighed in a sterile Petri dish and then transferred to a sterile mortar In the case of both brain and salivary gland tissue the material is ground up as previously indicated (see Annex 4) Whatever method is adopted for grinding the ground product is suspended in the diluent (normal saline with the addition of 2% inactivated horse or guinea pig serum) in the proportion of 1 part (by weight in grams) to 4 parts (by volume in millilitres) of diluent

The final result is a suspension which is centrifuged at low speed (1,000-2,000 revolutions per minute in a horizontal centrifuge) for 5 to 10 minutes or allowed to stand for several hours in the refrigerator

The supernatant is carefully decanted *without disturbing the sediment*, *it is a slightly* cloudy liquid without any visible particles and constitutes the so-called 20% suspension, which is the starting point for the virus dilution series

Preparation of Dilution Series

The test consists in making tenfold dilutions of the 20% solution and adding to each of the dilutions an equal quantity either of normal serum or of antirabies serum, the final result being a logarithmic dilution series of a 10% virus suspension neutralized with constant quantities of serum

The first step is therefore to prepare the virus dilution series

In order to do this, arrange three rows of 7 sterile tubes each The first row, placed in a rack separate from the other rows, comprises tubes with a capacity greater than 10 ml (ordinary 18 cm × 18 mm tubes) The second and third rows, which may conveniently be placed in the same rack, comprise tubes with a lower capacity (about 5 10 ml) which are shorter and whose internal diameter should be greater than that of the syringes used for inoculation If narrow 1 ml or 0 25 ml syringes of the "tuberculin type are used Kahn tubes and racks are perfectly satis factory for the second and third rows

Mark the first row of tubes with the numbers 1 to 7, using a grease pencil *In the same way, mark all the tubes in the second row with the* letter A (antiserum) and the final dilution titre 10^{-1}, 10^{-2} 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}

Mark the third row with the letter N (normal serum) and the dilution titres from 10^{-1} to 10^{-7}

Open all the tubes they *should remain open throughout the operation* and should only be re stoppered, their cotton wool plugs being kept for the purpose in a *sterile Petri dish* when they are placed in the incubator

It is advisable to place the tubes in the holes on the right side of the rack, leaving those on the left side empty

During the following processes, whenever a new mixture is added to a tube, that tube is displaced one place to the left Thus there is always an empty space between the last tube to which the mixture has been added and the following tube which has not been treated This avoids the risk of forgetting to treat one of the tubes, or of treating it twice

With a 5 ml or 10-ml pipette place a few ml of the 20% centrifuged virus suspension in the first tube on the left (marked 1) of the first row of tubes

Next place exactly 9 ml of diluent in each of the following tubes (marked 2 to 7) in the first row

With a sterile 2 ml pipette take from this first tube 1 6 ml of the 20% virus suspension, and place 0 3 ml of this in the first tube of each of the second and third rows and 1 ml in the second tube of the first row Discard the pipette with a clean pipette mix the contents of the second tube in the first row by repeatedly drawing up and expelling Next draw up 1 6 ml and place 0 3 ml in the second tube of each of the second and third rows and 1 ml in the third tube of the first row With another clean pipette, mix the contents of the third tube in the first row by repeatedly drawing up and expelling the liquid, and then place 0 3 ml in the third tube of each of the second and third rows and 1 ml in the fourth tube of the first row Continue in this manner until each of the tubes in the first row contains a 1/10 dilution of the preceding suspension while all the tubes in the second and third rows contain 0 3 ml of dilutions of virus ranging from 10^{-1} to 10^{-7}

Neutralization

Withdraw the first rack with all the tubes in the first row and discard them Add to all the tubes in the second row 0 3 ml of immune serum, and to all the tubes in the third row 0 3 ml of inactivated normal serum from the same species of animal Thus, a total of 2 1 ml of immune serum and 2 1 ml of normal serum will be used Mix by shaking the rack and incubate for 1 hour at 37 5°C in an ordinary incubator, shaking the tubes twice during incubation On removing them from the incubator, keep the virus serum mixtures at refrigerator temperature or in melting ice in a water-bath for 1 hour, then inoculate the mice intracerebrally

Inoculation of Mice

Eighty five previously untreated mice are kept in a glass or wire cage Arrange two rows of 7 jars each for the inoculated mice Commence inoculation with the highest dilution of virus neutralized by antiserum, this is the last tube on the right of the second row, marked 10^{-7}A Draw up the neutralized mixture into a sterile syringe, to ensure a homogeneous mixture and a tight fit of the piston, the mixture should be carefully drawn up and expelled several times, the point of the needle being kept

immersed in the liquid. Inoculate intracerebrally with the same dilution six mice, which are placed as they are inoculated in a jar labelled with the number of mice, the dilution (10^{-7}) of the neutralizing antirabies serum, and the date. Empty the syringe. With the same syringe and the same needle proceed in a similar fashion with the 10^{-6} neutralized dilution, taking care to rinse the syringe well with the new dilution, and continue with the 10^{-5} dilution, etc., finishing with the 10^{-1} dilution.

Change the syringe and needle, and follow the same procedure with the dilutions in the third row of tubes (normal serum), commencing with the highest dilution and finishing with the 10^{-1} dilution.

The mice are observed every day, their condition being entered in a notebook or on a card, using one page of the notebook or one card for each batch of six mice inoculated with each virus dilution and each serum. Keep the mice under observation for at least 30 days.

Calculation of End-Point

Using the Reed & Muench method,[1] tabulate the results as a function of the dilution. Add the results cumulatively (see Annex 6, page 264), doing this separately for mice which have received virus neutralized with the specific serum and for those which have received virus mixed with normal serum.

Interpretation of Results

Identification of the virus is confirmed if the antiserum results in a significant protection-rate, i.e., if there is sufficient difference between the number of deaths in the group treated with the normal serum and the number of deaths in the group treated with the antiserum.

Once the cumulative totals have been established, calculate the LD_{50} separately for the neutralized virus and for the mixture of virus and normal serum. The protection rate of the serum is given by the antilogarithm of the difference between the logarithm of the LD_{50} of the mice which have received the neutralizing serum and the logarithm of the LD_{50} of the mice which have received the mixture of virus and control serum. If there is no difference or if the antilogarithm is lower than 10, no neutralization has taken place and the virus is certainly not a rabies virus. If the difference between the two series of inoculated mice is represented by an antilogarithm between 10 and 49, the result is doubtful and the experi-

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[1] Reed, L. J. & Muench, H. (1938) *Amer. J. Hyg.* 27, 493

ment should be repeated If the difference is expressed by an antilogarithm of 50 or above (a logarithmic difference of 2 or more), neutralization is specific and it can be assumed that the virus is a rabies virus

Example

The unknown virus, in a dilution series of 10^{-1} to 10^{-7} with the antirabies serum A on the one hand and the antirabies serum N on the other hand, gives an LD_{50} of $10^{-2.25}$ in series A and 10^{-5} in series N

... between the two logarithms is $5 - 2.25 = 2.75$

... rate, is 57

... bies virus

In practice, a logarithmic differe ... fficient for the identification of a virus

NOTE

1 All the pipettes used in the test should be cotton plugged Each pipette is placed immediately after use in a receptacle which should be sent for sterilization at the end of the test In no case should contaminated pipettes be left on the benches or in temporary containers

2 Operators with a training in serology can reduce the quantities taken for the test described above by using only one 5 ml pipette and ten 1 ml serological pipettes with 0 01 ml calibrations In this case

(*a*) with the 5 ml pipette 2 7 ml of diluent are placed in tubes 2 to 7 of the first row (which may be of the same size as the tubes in rows 2 and 3)

(*b*) with the same pipette 0 3 ml of 20% virus suspension is placed in tube 1 With a 1 ml pipette, 0 3 ml is placed in tube 2 and 0 2 ml in the first tube of each of the second and third rows The liquid in tube 2 is mixed with a clean pipette 0 3 ml is placed in tube 3 and 0 2 ml in the second tube of each of the second and third rows Once the dilutions have been made 0 2 ml of antiserum is added to each of the tubes in the second row and 0 2 ml of normal serum to each of the tubes in the third row

3 If the titre of the experimental virus is already known from previous experiments, the number of log dilutions may usually be reduced to 5 The procedure is as above, but with three rows of 5 tubes each instead of 7 The total quantity of neutralizing serum and of normal serum employed is thus 1 ml for each serum

4 The same technique may be used for the titration of an antirabies serum in the presence of a known rabies virus, e g , fixed virus or street

virus of known titre Nevertheless, for accurate work, such as the titration of hyperimmune serum, it is preferable to use fixed quantities of virus with increasing dilutions of serum The procedure is as described above, but begins with the setting up of a log dilution series of the serum in the various tubes Once the dilution series has been set up, a constant volume of *rabies virus emulsion representing 200* LD_{50} is added to each tube, so that the final dilution of virus represents 100 LD_{50} By proceeding in this manner it is unnecessary to use more than one dilution of normal serum, which should be found to be without effect on the virus at a dilution of 10^{-1}

5 When working with a known rabies virus with a constant period of development in the mouse, the experiment may be stopped on the 20th day if the controls (normal virus serum mixture) have shown a normal *incubation period and a grouped mortality*

6 Half log dilutions In certain studies it may be useful to use dilutions closer to one another than the tenfold dilutions and to include half log dilutions in the experiment In this case, when preparing the usual tenfold dilutions of virus (1 ml of virus plus 9 ml of diluent), set up between the first and second row of tubes a supplementary row containing 2 16 ml of diluent in each tube At each transfer of the log dilutions, take up 2 ml instead of 1 ml and add 1 ml to the following tube (tenfold dilution) and

with a clean pipette and commencing with the highest half log dilution, carry out mixing with the same pipette in the same manner, proceeding through the range to the most concentrated dilution

Annex 6

DETERMINATION OF LD_{50} BY CUMULATIVE-TOTALS METHOD

At first sight it would seem logical to select as end point in a titration by animal inoculation either that titre which results in 100% mortality or that which results in 100% survival In fact, it can be shown that the region of titration least affected by statistical variations, systematic experimental errors, and accidents due to the abnormal resistance or sensitivity of the animal is that where half the animals die or show characteristic symptoms while the other half remain unaffected The titre at which

50% of the animals die is designated by the symbol LD_{50} This is the titre which we endeavour to determine by animal experiments

For observations with a significant statistical value, the method developed by Reed & Muench[1] is generally employed It is based on the following principles

1. Each group considered (inoculated animals, etc) should include at least five individuals

2. The critical dilution (50% point) should lie between at least two higher dilutions and two lower dilutions

3 The results are added up by the method of cumulative totals, consisting in adding for each dilution all animals which have died, starting from the highest titre (since they would have died at lower dilutions), and all animals which have survived, starting from the lowest titre (since they would have resisted higher dilutions)

Working in this way, results of the type shown in table I are obtained

TABLE I RESULTS OF MOUSE INOCULATIONS WITH DILUTION SERIES 10^{-1}–10^{-7}

Dilution	Number of mice			Cumulative totals		Mortality (%)
	Inoculated	dying	surviving	deaths	survivals	
10^{-1}	6	6	0	27	0	100
10^{-2}	6	6	0	21	0	100
10^{-3}	6	5	1	15	1	93
10^{-4}	6	5	1	10	2	83
10^{-5}	6	4	2	5	4	56
10^{-6}	6	1	5	1	9	10
10^{-7}	6	0	6	0	15	0

It is clear that, in table I, the dilution which would have given exactly 50% mortality lies between the dilution 10^{-5} (56% mortality) and the dilution 10^{-6} (10% mortality)

[1] Reed L J & Muench, H (1938) *Amer J Hyg* 27, 493

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10^{-3}	6	5	1	15	1	93
10^{-4}	6	5	1	10	2	83
10^{-5}	6	4	2	5	4	56
10^{-6}	6	1	5	1	9	10
10^{-7}	6	0	6	0	15	0

It is clear that, in table I, the dilution which would have given exactly 50% mortality lies between the dilution 10^{-5} (56% mortality) and the dilution 10^{-6} (10% mortality)

---

[1] Reed L J & Muench H (1938) *Amer J Hyg* 27 493

This dilution could be determined experimentally by repeating the experiment with a range of dilutions between 10^{-5} and 10^{-6}. To save time and economize in animals, the 50% point is calculated by one of the following methods.

Arithmetical methods

Method A Let us consider the dilutions on either side of the 50% point which is to be determined. If A is the number of animals dying at the higher concentration (lower dilution) and B that of the mice surviving at the same concentration (A > B, if A equalled B, the titre would be exactly that of the LD_{50} point), and if C is the number of animals dying, and D the number surviving, at the less concentrated of the two dilutions (C < D) the factor to be added to the [illegible]

$$\frac{(A - B)(C + D)}{2(AD - BC)}$$

The dilutions on either side of the LD_{50} dose and their results, i.e.,

$$\frac{A \; B}{C \,|\, D}$$

are, for the dilutions 10^{-5} and 10^{-6} given in table I,

$$\frac{5 \,|\, 4}{1 \,|\, 9}$$

Using the formula, the result is

$$\frac{(5 - 4)(1 + 9)}{2(45 - 4)} = \frac{10}{82} = 0.12$$

The LD_{60} dilution is therefore $10^{-5.12}$

Method B If the mortality percentages have been calculated, the 50% point may be determined with sufficient accuracy by using the following formula

$$\frac{(\text{mortality immediately above } 50\%) - 50}{(\text{mortality immediately above } 50\%) - (\text{mortality immediately below } 50\%)}$$

In the preceding example, where the mortality rates on either side of the 50% dilution are respectively 56% and 10%, this formula gives

$$\frac{56 - 50}{56 - 10} = \frac{6}{46} = 0.12$$

Graphic method

The same result can be derived without any calculation by making use of a sheet of semi logarithmic paper with decimal divisions It is sufficient to know the cumulative totals of the mice surviving and dying at the doses on either side of the 50% point (the values A, B, C, and D in the arithmetical method) The number of mice is indicated on the arithmetic scale of the sheet as an ordinate, the virus dilution being entered on the logarithmic scale as an abscissa The four points corresponding to the mice surviving and mice dying are plotted in this way against the corresponding concentrations, which are entered on the logarithmic scale, the higher concentration being on the left and the higher dilution of the two log dilutions on either side of the 50% point being on the right The figures for the number of mice dying and the number surviving are joined by straight lines The point of intersection of the two lines gives the 50% titre, the value of which is read off on the logarithmic scale

Probit method[2, 3]

This method, which is a variant of the preceding one, consists in ascertaining the 50% dose in probability units, or probits On plotting as ordinates the number of animals dying at virus doses varying according to a function shown as abscissae, a typical S shaped curve is obtained, which becomes, when the doses used are expressed logarithmically, a straight line showing the response probability of a homogeneous population to any dose between the limits considered The response may be found either by using special graph-paper (Bonét-Maury) or by referring to the tables in Finney s book on probit analysis[4]

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[2] Bliss C I (1938) *Quart J Pharm* 11, 192
[3] Bonét Maury P (1942) *Ann Inst Pasteur* 68 491
[4] Finney D J (1947) *Probit analysis a statistical treatment of the sigmoid response curve* Cambridge

DISCUSSION. PART V

The vaccination of patients bitten by animals suspected of having rabies was discussed It was mentioned that in Tunisia, it was not considered desirable to defer treatment of patients until symptoms of the disease appeared in the animal, and that it should not be forgotten that in some countries the chances of being infected with rabies were rather high In Tunisia it was proposed that treatment should be started immediately after the bite, and that it should be continued whether or not the dog showed symptoms The discussion leader stated that human vaccination measures to be used in connexion with rabies had to be adapted to local conditions One could not disagree with the Tunisian method of starting the inoculation of bitten patients immediately in a country where the incidence of rabies was high A question was asked as to whether a vaccinated dog which had been bitten by a rabid animal could harbour the virus to such an extent that it might transmit the disease without being clinically ill In reply, it was stated that it had never been conclusively proved that a vaccinated dog could act as a transmitter of the disease without showing some clinical symptoms

The question of paralyses in connexion with vaccination was raised, and it was asserted that experience varied somewhat in different countries, with the racial factor perhaps playing some part In discussing this problem, the discussion leaders felt that the vaccine and the technique applied must be up to standard, sensitivity to some factor in the nerve substance was the probable explanation for paralyses following vaccination They were not convinced that the statistics available always gave the true picture of the situation, and the role of racial factors in this connexion was rather difficult to substantiate In reply to an inquiry as to whether the serum-vaccine method would lessen the frequency of paralyses, the discussion leader stated that the use of serum might lower the number of vaccine inoculations required, which in turn would lessen the chances of paralytic accidents, since these accidents usually occurred after the seventh inoculation of vaccine Work in this connexion was now under way in several laboratories

It was stated that in Morocco vaccination of cattle had also been carried out with satisfactory results Four injections of 80 ml of vaccine were given at 48 hour intervals for an animal of 250-kg weight The discussion leader pointed out, however, that no carefully controlled post-exposure vaccination experiments in cattle had as yet, been carried out, so that such use of vaccine was still on a purely empirical basis

The question of the number of rabies vaccine injections to be given to dogs was also discussed In the USA only one injection of a potency tested vaccine is usually given to dogs, and field and laboratory results show that one inoculation gives a high degree of protection for at least one to two years As regards cattle exposed to rabies, preliminary experiments indicate that a combined serum and vaccine treatment might be the solution

Experience in the United Kingdom of Great Britain and Northern Ireland with respect to a six month quarantine period for dogs entering the country had been very satisfactory, although it was pointed out that, in two out of eight dogs developing rabies while being held in quarantine, the disease developed after the expiration of the six month period This period however had proved sufficient from a practical point of view

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JOINT WHO/FAO SEMINAR ON ZOONOSES

Vienna, 24 29 November 1952

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